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STUDIES OF ETHANOL METABOLISM IN GERMINATING CASTOR BEAN ENDOSPERM

by

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A THESIS

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OF MASTER OF SCIENCE

DEPARTMENT OF BOTANY

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FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled STUDIES OF ETHANOL METABOLISM IN GERMINATING CASTOR BEAN ENDOSPERM submitted by Carol Anne Peterson in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

The role of acetate and the glyoxylate cycle in ethanol metabolism by castor bean endosperm has been examined.

Endosperm tissues were found to metabolize ethanol-C^{14} during the early stages of germination and seedling development. Over this period, the tissues also contained an active alcohol dehydrogenase system.

Properties of this dehydrogenase system including the effects of substrate concentration, enzyme concentration, coenzyme requirements, specificity, reversibility, effects of iodoacetate and intracellular localization have been studied.

The kinetics of ethanol-1-C^{14} and ethanol-2-C^{14} utilization by tissue slices of germinating endosperm have been examined over periods of ethanol metabolism ranging from 60 seconds to 4 hours. These studies have indicated that the organic acids, particularly malate are the primary products of ethanol metabolism.

Isotopic dilution studies using acetate have been carried out to assess the importance of this compound as an intermediate in ethanol metabolism. The results are interpreted as indicating that ethanol oxidation involves the intermediary formation of acetate and acetyl CoA.

Studies on the role of the glyoxylate cycle during ethanol metabolism have indicated a major role of malate

synthetase in this metabolism. The metabolic products derived from the individual carbon atoms of ethanol also shows that the partial reactions of the glyoxylate cycle represent an important pathway for ethanol metabolism in this tissue.

Comparative studies of ethanol and acetate metabolism using 5 and 7 day old castor bean endosperm tissues and excised corn root tips have revealed certain basic differences in the products formed from these compounds. In all cases large amounts of ethanol carbon were involved in lipid synthesis.

The possible pathways for the utilization of ethanol and acetate in these tissues have been discussed.

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INTRODUCTION AND LITERATURE REVIEW

The role of ethanol in plant metabolism has been a controversial issue since the discovery of its formation in plants (Le Chartier and Bellamy 1869). Pfeffer (1910) proposed that the formation of ethanol was essentially an intermediary stage in hexose breakdown but this view found little support from later research. Kostychev (1927) concluded from extensive analytical experiments that ethanol was very slightly, if at all, metabolized by higher plant tissues. In agreement with this finding, Thomas (1925) also obtained evidence that in apple tissues, ethanol cannot be metabolized. For example, he found that ethanol concentrations accumulating in apple fruits during a period of anaerobiosis, were not decreased during a subsequent exposure to air, nor did the acetaldehyde content of this tissue increase. Thomas' original view that ethanol was produced as a result of anaerobic conditions from a normal glycolytic intermediate is now generally accepted.

Higher plants often undergo a short period of natural anaerobiosis during the early stages of germination. Ethanol and lactic acid commonly accumulate during this period until breakage of the testa returns the plant to aerobic conditions (James 1953, Cossins and Turner 1959 and Cossins 1964). Whether or not ethanol could be metabolized under aerobic conditions was, however, questionable. Most experimental evidence prior to 1962 indicated that it could not. For example, Lowe and James (1960) found no incorporation of C^{14} into plant acids

and concluded that ethanol was not catabolized in carrot root slices despite the presence of an active alcohol dehydrogenase.

However, Cossins and Turner (1962) found that during germination, seeds of peas, runner bean, broad bean, castor bean and wheat all lost more alcohol than was accounted for by losses due to volatility. They were able to correlate the alcohol dehydrogenase activity in the seeds with the quantity of the unrecovered ethanol. Another report by Cossins and Turner (1963) confirmed their earlier results and added further evidence for the aerobic utilization of ethanol in germinating peas. The amount of ethanol lost increased when the testas of the seeds were wholly or partially removed thereby allowing the tissue to become more aerobic. Ethanol feeding was accompanied by increases in the O_2 uptake, decreases in the respiratory quotient and accumulations of acetaldehyde. The same effects on the gaseous exchange was observed when the testas were removed. Feeding experiments employing ethanol-2-C^{14} showed that the ethanol molecule was rapidly incorporated into acetaldehyde, acetone and various organic and amino acids. In a later publication, Cossins and Beevers (1963) demonstrated that ethanol utilization is of widespread occurrence in higher plants. From ethanol-C^{14} feeding experiments it was shown that C^{14} was incorporated into every major fraction of carrot discs, castor bean endosperm tissue, corn coleoptiles, pea shoots, corn shoots and apple tissue.

It has long been known that many microorganisms can

utilize ethanol (Fruton and Simmonds 1960). In many species this compound can be utilized as the sole source of carbon for growth of the organism. Many algae can utilize ethanol, Polytomella coeca, Chilomonas paramecium, Prototheca, Tribonema aequale and Euglena bacillaris among them. The presence of ethanol is now known to enhance the growth of Chlorella vulgaris, Coccomyces, Scenedesmus and Euglena and so casts doubt on the validity of early experimental work in which growth substances dissolved in ethanol were used. The bacteria Pseudomonas and Escherichia coli as well as many moulds (eg. Rhizopus) are able to utilize ethanol as their sole source of carbon (Lewin 1962).

It is generally agreed that ethanol formation or oxidation might occur via a reversible alcohol dehydrogenase system. This enzyme has been extracted from many plant sources and its properties studied (Stafford and Vennesland 1953, App and Meiss 1958, Racker 1950 and Cossins and Turner 1962). The alcohol dehydrogenase system extracted from wheat germ (Stafford and Vennesland 1953) was found to catalyze the reduction of nicotinamide adenine dinucleotide (NAD) and to a lesser degree, nicotinamide adenine dinucleotide phosphate (NADP) in the presence of ethanol. The reaction was readily reversed in the presence of acetaldehyde. The ability of the alcohol dehydrogenase to utilize both NAD and NADP suggested that there may be more than one alcohol dehydrogenase present in wheat germ tissue. Alcohol dehydrogenase activity was completely

inhibited by 10^{-2} M iodoacetate and is therefore presumed to contain sulphydryl groups for its activity.

Since rice will grow in both aerobic and anaerobic conditions, the effects of these environments on ethanol formation and alcohol dehydrogenase activity can be directly compared using this tissue. The alcohol dehydrogenase present in rice coleoptiles was shown to be an adaptive enzyme (App and Meiss 1958). When one week old anerobically grown coleoptiles were aerated, their alcohol dehydrogenase activity immediately decreased. The enzyme activity could be restored by returning the seedlings to anaerobic conditions. Ethanol at concentrations less than 10^{-1} M was found to induce enzyme activity while additions of acetaldehyde would not affect it.

Hageman and Flesher (1960), using the alcohol dehydrogenase extracted from corn seedlings, also found it to be adaptive to anaerobic conditions. The inducer in this case was found to be acetaldehyde, not ethanol.

Cossins and Turner (1962) found that the presence of iodoacetate, at concentrations greater than 10^{-3} M, completely inhibited the activity of alcohol dehydrogenase obtained from germinating pea seeds. Optimum enzyme activity was found to occur using a phosphate medium at a pH of 8.04 with a concentration of 2.00 mgm NAD and ethanol as a substrate.

Ethanol metabolism has undergone considerable investigation in animal tissues, particularly in rat liver. The first step in the oxidation of ethanol in this tissue could conceivably

be due to the action of either an alcohol dehydrogenase system or a coupled oxidation with catalase since both enzymes are known to be present. Keilin and Hartree (1945) found that additions of ethanol in the presence of catalase doubled the oxygen uptake. Furthermore crystalline catalase added to the system caused the oxidation of ethanol to acetic acid. They concluded that catalase activity might play a significant role in ethanol oxidation in animal tissues. This idea has, however, received little support from later work with intact rats and rat liver slices. Results from studies using 3-amino-1,2,4-triazole as an inhibitor of catalase (Kinard, Nelson and Hay 1956) and o-phenanthroline (Lundquist, Svendsen and Peterson 1963) as an inhibitor of alcohol dehydrogenase have indicated that ethanol oxidation *in vivo* occurs via an alcohol system.

In plant tissues, the first step in ethanol metabolism is probably its oxidation to acetaldehyde. However, little is known about the metabolic pathways by which this oxidation product is utilized. During the course of the present investigation, Castelfranco, Bianchetti and Marré (1963) published a comparative study of ethanol and acetate metabolism in potato tuber slices and pea stem sections. The percentage of added substrate incorporated into the lipid, CO_2 and the 80% ethanol-soluble fractions from ethanol-1-C^{14}, ethanol-2-C^{14}, acetate-1-C^{14} and acetate-2-C^{14} was determined. Ethanol was found to be more readily incorporated into the lipid fraction

than acetate. Also differences in the distribution of C^{14} in the various fractions isolated indicated basic differences in ethanol and acetate metabolism.

Studies on ethanol metabolism in animals have been fairly extensive recently, since this compound is thought to be associated with a fatty liver condition in animals. Nikkila and Ojala (1963) found that the administration of ethanol to intact rats caused an increase in the L-glycerophosphate content of the liver a few hours after treatment. This, they conclude, might be due to increases in $NADH_2$ resulting from ethanol oxidation which could be utilized in the reduction of dihydroxyacetone phosphate. Palmitic acid-C^{14} added to the blood stream was found to have an increased uptake into the livers of ethanol-treated rats. The authors conclude that a product of the ethanol metabolism causes metabolic alterations in the liver which will eventually bring about its fatty condition. Jones, Losowsky, Davidson and Lieber (1963) concluded from their studies on the effect of ethanol on the composition of the human blood plasma that ethanol was a direct agent in lipid formation. Clearly, if ethanol is the cause of fatty liver, the metabolic pathways by which ethanol and acetate are metabolized in these tissues should be different. This conclusion is not completely substantiated by recent experiments using ethanol-C^{14} and acetate-C^{14}. For example, Lundquist, Svendsen, and Peterson (1963) have presented evidence that ethanol supplied to rat liver suspensions was converted almost quanti-

tatively to acetate. Dajani and Orten (1962) discovered that ethanol-C^{14} was utilized more readily than acetate in rats kept on a diet including ethanol whereas the reverse is true for the normal rat. This effect was probably due to changes in the alcohol dehydrogenase activity of the treated animals. In a later publication, Dajani, Danielski and Orten (1963) confirmed this by demonstrating increasing levels of alcohol dehydrogenase activity in the livers of ethanol-treated rats. This trend was also observed to a lesser degree in the blood sera of the test animals.

To determine whether ethanol is metabolized exclusively via acetate, Smith (1961) used diabetic rats since this condition is associated with impaired acetate metabolism. The incorporation of ethanol-C^{14} and acetate-C^{14} into liver slices of the diabetic animals was severely and equally depressed. Isotopic competition studies also indicated that ethanol was metabolized via acetate.

The most recent attempt to determine the metabolic fate of ethanol in animal tissues has been made by Russell and van Bruggen (1964) using labelling ratios. If these substrates are metabolized by the same pathway, the ratio for the incorporation of acetate-1-C^{14} to acetate-2-C^{14} into any particular compound should be equal to that obtained from ethanol-1-C^{14} and ethanol-2-C^{14}. This rationale should be correct even if there is a preferential incorporation of one of the substrates. Similarity of these ratios was found in choelesterol and the fatty acids extracted from various organs of the experimental

animals. The ratios for acetyl sulphanilamide were also the same. These results might therefore indicate certain similarities in the pathway by which ethanol and acetate are metabolized in rat tissue.

Extensive feeding experiments using a variety of higher plant tissues (Cossins and Beevers 1963) have indicated that ethanol is probably oxidized to acetate and converted to acetyl CoA. The metabolism of acetyl CoA by plant tissues probably depends on physiological changes occurring within the tissues examined (Beevers 1961). For example, during the germination of fatty seeds such as castor bean, acetyl CoA arising from fatty acid oxidation might be utilized via the glyoxylate cycle (Beevers 1961). This cycle allows for a net synthesis of important cellular components from acetyl CoA. Enzymes necessary to catalyze the partial reactions of this cycle are of fairly widespread occurrence in micro-organisms (Kornberg and Elsden 1961) and are particularly active in tissues converting fat to carbohydrate (Carpenter and Beevers 1959, Yamamoto and Beevers 1960 and Bradbeer and Stumpf 1959). The glyoxylate cycle can be regarded as a modification of the TCA cycle but differs from the latter by being essentially synthetic. The operation of the cycle results in the formation of a four-carbon acid from two acetyl CoA molecules as outlined in scheme (i). If ethanol is converted to acetyl CoA in tissues containing an active glyoxylate cycle, this might represent a major pathway for ethanol utilization.

SCHEME (i)

Reactions of the Glyoxylate Cycle

* Carbon atoms derived from acetyl-2-C^{14} CoA entering by reaction (1).

● Carbon atoms derived from acetyl-2-C^{14} CoA entering by reaction (5).

Enzymes catalyzing the reactions are:

(1) condensing enzyme

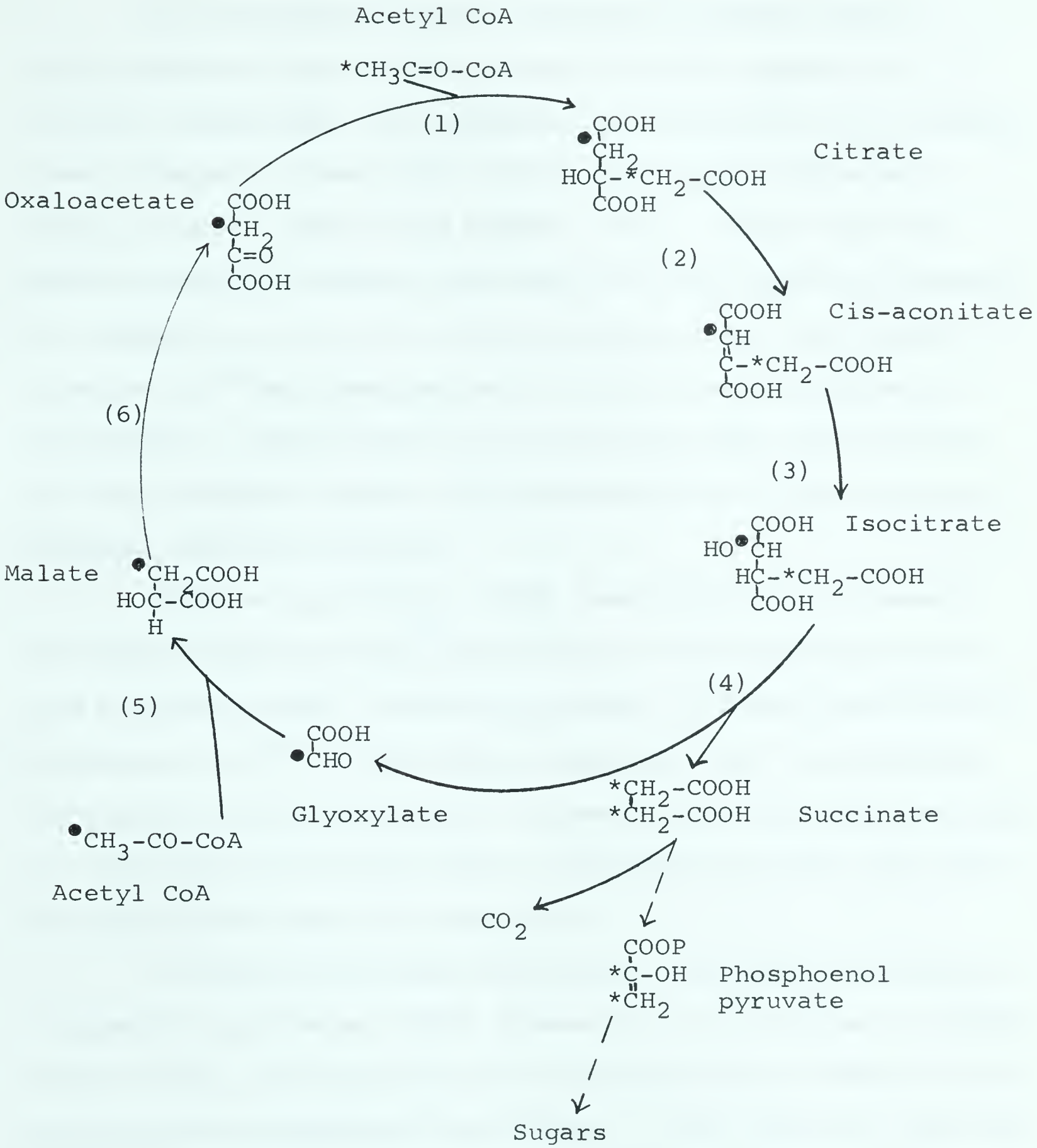
(2) aconitase

(3) aconitase

(4) isocitrase

(5) malic synthetase

(6) malic dehydrogenase


Acetyl CoA
*CH3C=O-CoA
(1)
COOH
CH2
HOC-*CH2-COOH
COOH
Citrate
Oxaloacetate
COOH
CH2
C=O
COOH
(2)
COOH
CH
C-*CH2-COOH
COOH
Cis-aconitate
(3)
COOH
HO CH
HC-*CH2-COOH
COOH
Isocitrate
(4)
(5)
(6)
Malate
CH2COOH
HOC-COOH
H
COOH
CHO
Glyoxylate
*CH2-COOH
*CH2-COOH
Succinate
CH3-CO-CoA
Acetyl CoA
CO2
COOP
*C-OH
*CH2
Phosphoenol
pyruvate
Sugars

In the tissues studied, succinate produced during cycle operation was readily converted into a variety of cellular components. For example, acetate-1-C^{14} fed to castor bean endosperm slices first labels the organic acids and later, sucrose (Canvin and Beevers 1961). Label from the methyl carbon of acetate was found to occur almost exclusively in carbons 1, 2, 5, and 6 of the glucose moiety of sucrose. Acetate-1-C^{14} was incorporated into the 3 and 4 carbons of the hexose. These results are consistent with the operation of the glyoxylate cycle, with randomization of label occurring through fumarase activity.

Bradbeer and Stumpf (1959) demonstrated the presence of malate synthetase and isocitritase in germinating peanut and sunflower seeds. Intact cotyledons utilized acetate-1-C^{14} and acetate-2-C^{14} in the dark, essentially for the synthesis of organic acids and sugars. Intramolecular labelling patterns in the products produced were in agreement with the view that the glyoxylate cycle was operating.

A survey of the distribution of isocitritase in plants (Carpenter and Beevers 1959) showed that this enzyme is widely distributed, but fatty tissues were consistently found to contain greatest amounts of this enzyme. High levels of isocitritase were detected in 5 day old castor bean endosperm, but the enzyme was not found in other parts of the seedling. Similar studies on malate synthetase (Yamamoto and Beevers 1960) have also indicated that fatty tissues such as castor bean endosperm contain large amounts of this enzyme.

An indication of the pathway by which four carbon acids arising from the glyoxylate cycle are incorporated into sugar by castor bean endosperm tissues was obtained from studies using pyruvate-C^{14} (Neal and Beevers 1960). The experiments indicated that carbons 2 and 3 of pyruvate were incorporated into the organic acids via the glyoxylate cycle. Reversal of glycolysis leading to sucrose formation apparently did not involve pyruvate as an intermediate. Benedict and Beevers (1961) conclude that the enzyme system most likely to be involved in the conversion of dicarboxylic acids to an intermediate of glycolysis was PEP carboxykinase, catalyzing the following reaction:

$$\text{oxaloacetate} + \text{ATP} \overset{Mg^{++}}{\rightleftharpoons} \text{ADP} + CO_2 + \text{phosphoenolpyruvate}$$

The incorporation of acetate-1-C^{14} and acetate-2-C^{14} into CO_2, sugars, organic acids and amino acids by castor bean endosperm slices has been studied in some detail (Canvin and Beevers 1961). Data obtained from time course experiments, intramolecular labelling patterns and the products derived from the individual carbons of acetate indicated that the glyoxylate cycle was operating.

Stiller, Neal and Beevers (1958) demonstrated an active CO_2 fixation system in the castor bean endosperm tissue. $C^{14}O_2$ supplied to tissue slices was incorporated mainly into malate and sucrose. Benedict and Beevers (1961) explain these results by an exchange reaction. $C^{14}O_2$ undergoes a condensation with PEP, catalyzed by PEP carboxylase or PEP carboxykinase,

and mixes with the malate pool via oxaloacetate. CO_2 from this pool is then given off by the action of PEP carboxykinase. The tissue does not gain any carbon by this mechanism but some label originally in the $C^{14}O_2$ can spread to malate and sucrose by this pathway.

Considerable data on metabolic events occurring in the castor bean endosperm tissue have accumulated during the past decade. The present studies have therefore been designed to determine whether ethanol is metabolized by these tissues by a pathway involving conversion to acetate and acetyl CoA. In this work, details of the metabolic pathway for ethanol metabolism were examined in an attempt to determine the possible role of the glyoxylate cycle during ethanol metabolism.

MATERIALS AND METHODS

PLANT MATERIALS:

Seeds of Ricinus communis L. var. zanzibarensis were soaked for 24 hours in distilled water at 25°C prior to planting in moist Vermiculite. They were then germinated at 25° C in the dark for periods up to 7 days. For a comparative study of ethanol and acetate metabolism in corn root tips, seeds of Zea mays L. cv. 'Falconer' were soaked and germinated as above for a period of 3 days, the radicles at this time having a length of 1.5 cm.

EXPERIMENTAL METHODS:

1) Assay of Alcohol Dehydrogenase Activity:

Extracts containing alcohol dehydrogenase were prepared from castor bean endosperm tissue at various stages of germination. Slices of endosperm (5 gm f wt) were chilled to 2°C before grinding with 10 ml of ice-cold buffer (0.1 M Na_2HPO_4, pH 8.0) in a chilled mortar. The homogenate was passed through four layers of cheesecloth and centrifuged at 10,000 g for 20 min at 2°C to remove all cell debris. The volume of the supernatant was adjusted to 30 ml by the addition of ice-cold 0.1 M Na_2HPO_4 solution (pH 8.0) and maintained at 2°C prior to enzyme assay. Alcohol dehydrogenase activity was assayed by measuring the reduction of NAD in a Beckman DB Spectrophotometer according to the method described by Racker (1950). The sample cuvette, having a 1 cm light path, contained 50 μmoles NAD, 300 μmoles ethanol, and 0.1 ml

enzyme (containing approximately 1 mg protein) in a total volume of 3 ml. The control cell contained all the above reagents except ethanol. Enzyme activity was measured by the initial reaction velocity, calculated from the increase in optical density at 340 mμ, occurring between the 15 and 45 second readings. The results of these assays were expressed in units of alcohol dehydrogenase activity, one unit being defined as the amount of enzyme causing a change in optical density of 0.001 per minute. Specific enzyme activity was expressed as units per gm f wt, per gm dry wt, and per mgm protein.

Alternatively, alcohol dehydrogenase activity of the extracts was assayed by the rate of decolourization of methylene blue using the Thunberg tube technique (Burris, 1959). In this procedure, the main tube contained 1.5 mmoles ethanol, 150 μmoles phosphate buffer (Na_2HPO_4, pH 8.0), 0.15 μmoles of methylene blue, and 0.34 μmoles nicotinamide adenine dinucleotide (NAD). One ml of enzyme was added to the side-arm. The tubes were evacuated and incubated for 5 min at 25°C to allow for temperature equilibrium before mixing. A blank, containing all the components except ethanol, was included in each determination. The time taken for dye decolourization after mixing was measured. Enzyme activity was expressed as the amount of methylene blue (in μl) reduced per hour. Specific enzyme activity was expressed as microliters of methylene blue reduced in one hour per gm f wt, per gm dry wt, and per mgm protein.

Protein was determined spectrophotometrically by the method of Warburg and Christian (1941). The optical density of the homogenates was determined at 280 mμ and 260 mμ. The ratio of the absorbance values of the sample at 280 mμ and 260 mμ was used in determination of the protein concentration in mg/ml (Layne, 1960).

In order to determine the possible intracellular localization of the alcohol dehydrogenase present in the endosperm tissue, mitochondrial and cytoplasmic fractions were separated by the method of Beevers and Walker (1956). The testas of 5 day old castor beans were removed and the endosperm chilled to 2°C prior to grinding with sand in a chilled mortar with ice-cold 0.5 M sucrose, 0.02 M magnesium chloride and 0.1 M phosphate buffer (NaH_2PO_4) pH 7.0. One ml of the grinding solution was used per gram of endosperm tissue. The homogenate was passed through four layers of cheesecloth and centrifuged at 10,000 g for 10 minutes at 2°C to remove unbroken cells and cell wall fragments. The supernatant was then centrifuged at 18,000 g for 30 minutes at 2°C to sediment the mitochondria. The fat pellet was discarded, and the supernatant (cytoplasmic fraction) decanted and kept at 2°C. The mitochondria were then washed by resuspension in a small volume of the grinding medium and resedimented by centrifugation at 18,000 g for 30 minutes. The mitochondrial fraction was then finally suspended in 1 ml cold phosphate buffer (0.1 M Na_2HPO_4 pH 5.5). The alcohol dehydrogenase activity in the cytoplasmic

and mitochondrial fractions was assayed by spectrophotometric methods as described previously.

2) Feeding Experiments Using Ethanol-C^{14} and Acetate-C^{14}:

Ethanol-1-C^{14}, ethanol-2-C^{14}, acetate-1-C^{14}, and acetate-2-C^{14} were purchased from Atomic Energy of Canada Limited, Ottawa and from the California Corporation for Biochemical Research, Los Angeles. The radioactive stock solution from the purchaser was diluted with ice-cold distilled water to give specific activities within the range of 0.5 µc per µmole to 10 µc per µmole. In experiments where ethanol-C^{14} and acetate-C^{14} solutions of equal specific activities were used, the stock solutions were diluted with solutions containing micromolar amounts of ethanol and acetate respectively.

To facilitate the penetration of added metabolites, the castor bean endosperm tissue was cut into slices about 1 mm thick using a sharp razor blade. All samples were washed three times with distilled water before being used. Tissue slices (0.5 gm f wt) were placed in Warburg flasks (capacity 17 ml) and 0.2 ml of 20% KOH solution was added to the centre wells to absorb CO_2 released during the experimental periods. When a larger tissue sample was employed, 1 gm of tissue slices was placed in an Erlenmeyer flask (capacity 70 ml) fitted with a centre well which contained 2 ml of 20% KOH to absorb CO_2. In experiments in which acetate and ethanol metabolism were compared, the tissue slices were incubated with 50 µmoles of phosphate

buffer at pH 5.5.

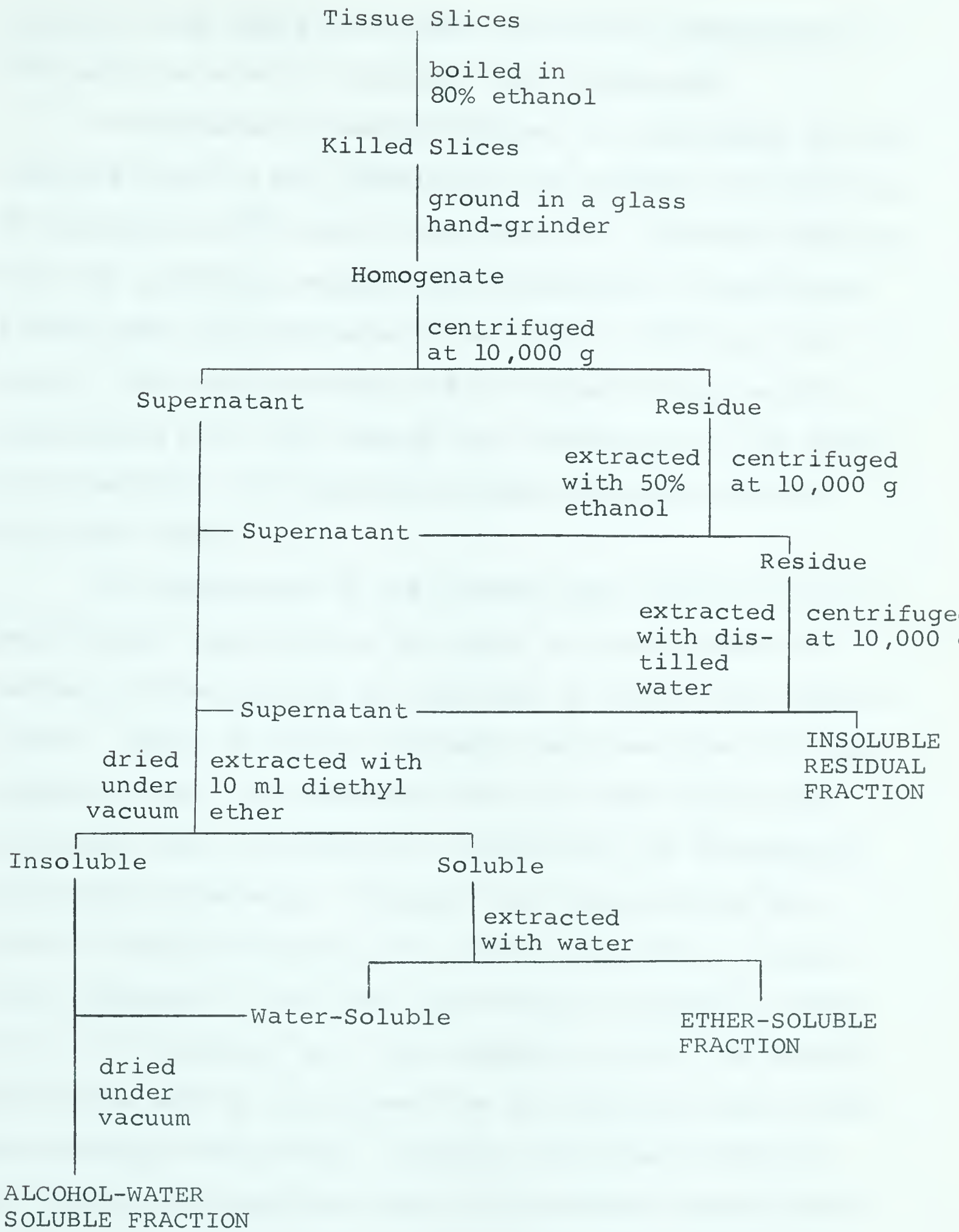
In all feeding experiments, the radioactive solutions were added to the slices by means of a microsyringe, the final volume of liquid in the flasks being sufficient to maintain the tissue in a moist condition. The flasks were connected to conventional Warburg manometers and incubated for periods of 5 to 360 minutes at 30°C. In experiments where Erlenmeyer flasks were used, these were sealed with a serum stopper and incubated for periods ranging from 5 to 240 minutes. In experiments designed to follow the release of $C^{14}O_2$ during the experimental period, the KOH solution was changed via a microsyringe needle introduced through the serum stopper. In all cases, the $C^{14}O_2$ absorbed by the KOH solution was withdrawn and converted to $BaC^{14}O_3$ by addition of excess $Ba(OH)_2$ before the slices were killed by boiling in 80% ethanol for 1 minute. In order to determine the distribution of C^{14} in the products of ethanol-C^{14} and acetate-C^{14} metabolism, the tissues were extracted and the alcohol/water soluble extract fractionated by the procedure outlined below.

3) Analytical Procedures:

Following killing in boiling ethanol, the tissues were extracted and separated into three fractions; (a) ether-soluble materials (mainly lipids), (b) insoluble residue (mainly cellulose and insoluble proteins), and (c) alcohol/water soluble materials (see Figure 1). Centrifugation was carried out in a Servall Refrigerated -

FIGURE 1.

Procedure for Fractionation of Castor Bean Endosperm Tissue Slices


Tissue Slices
boiled in 80% ethanol
Killed Slices
ground in a glass hand-grinder
Homogenate
centrifuged at 10,000 g
Supernatant
Residue
extracted with 50% ethanol
centrifuged at 10,000 g
Supernatant
Residue
extracted with distilled water
centrifuged at 10,000 g
Supernatant
INSOLUBLE RESIDUAL FRACTION
dried under vacuum
extracted with 10 ml diethyl ether
Insoluble
Soluble
extracted with water
Water-Soluble
ETHER-SOLUBLE FRACTION
dried under vacuum
ALCOHOL-WATER SOLUBLE FRACTION

Automatic High Speed Centrifuge and drying accomplished at 40°C under vacuum in a Buchler Flash Evaporator.

The insoluble residue obtained in this manner was hydrolyzed with 6 N HCl containing 0.1% stannous chloride for 20 minutes at 112°C and 15 lbs pressure. Following hydrolysis, the insoluble residue was collected on a glass-fibre filter paper disc and dried in an oven at 100°C for six hours. The dried material and an aliquot of the soluble hydrolysate were then assayed for radioactivity (see below) and constitute the insoluble residual fraction referred to in the tables.

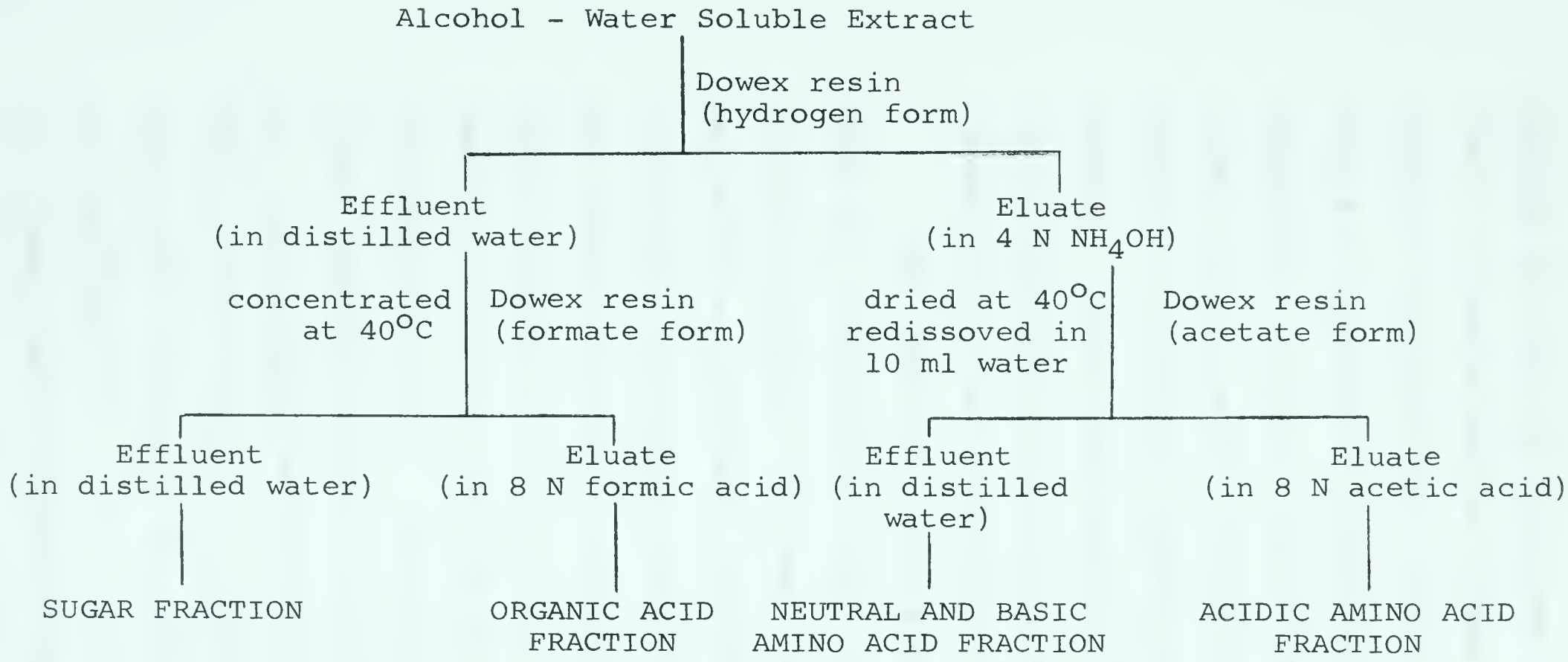
The components of the alcohol/water soluble fraction were further separated on the basis of charge using ion exchange chromatography as described by Cossins and Beevers (1963). Dowex AG 50W-X8 (hydrogen form) and Dowex AG 1X10 (chloride form) ion exchange resins (200-400 mesh) were purchased from the California Corporation for Biochemical Research, Los Angeles. Columns were prepared from the chloride form by plugging the constricted end of a glass tube (diameter 1.5 cm) with glass wool and adding a water slurry of the resin to a final depth of 4 cm. The column was washed with a continuous flow of distilled water until the effluent was neutral. Acetate and formate forms of the resin were generated from the purchased chloride form by passing 1 M solutions of sodium acetate or sodium formate through a column of the resin until the effluent was chloride-free (as indicated by the silver nitrate test).

The chloride-free resin was then washed with 40 ml of 0.1 M acetic acid (or 0.1 M formic acid in the case of the formate resin) before being neutralized by washing with distilled water. Components of the alcohol/water soluble fraction were separated using these resins according to Figure 2. An aqueous solution of the mixture to be fractionated was introduced to the top of the column. Materials not adsorbed by the resin were washed through with 40 ml of distilled water. Adsorbed materials were then displaced by addition of 30 mls of the eluting solutions as shown in Figure 2.

Separation of the individual acids present in the organic acid fraction was accomplished by gradient elution using the method described by Palmer (1955). The organic acid fraction, prepared as described above, was dried to remove traces of formic acid, dissolved in 10 ml of distilled water and added to a 1 x 11 cm column of Dowex formate resin (generated as outlined above). Individual acids present in this fraction were eluted from the resin using an increasing concentration of formic acid. Formic acid (8 N) was passed from an upper reservoir into a lower one containing 500 ml of distilled water so that the outflow of the lower solution was balanced by the inflow of formic acid. The lower solution was mixed by means of a magnetic stirrer. The effluent from the column was collected in 2.5 ml fractions by means of a Buchler Fraction Collector equipped with an automatic volumetric dispensing head.

FIGURE 2.

Ion Exchange Chromatography of Castor Bean Endosperm Extracts


Alcohol - Water Soluble Extract
Dowex resin
(hydrogen form)
Effluent
(in distilled water)
Eluate
(in 4 N NH_4OH)
concentrated
at 40°C
Dowex resin
(formate form)
dried at 40°C
redissoved in
10 ml water
Dowex resin
(acetate form)
Effluent
(in distilled water)
Eluate
(in 8 N formic acid)
Effluent
(in distilled
water)
Eluate
(in 8 N acetic acid)
SUGAR FRACTION
ORGANIC ACID
FRACTION
NEUTRAL AND BASIC
AMINO ACID FRACTION
ACIDIC AMINO ACID
FRACTION

Between 100 and 150 fractions (2.5 ml in each) were routinely collected and an aliquot of each was removed for assay of radioactivity. Since different organic acids were eluted at different concentrations of formic acid, the radioactivity in the various fractions corresponded to individual organic acids. Thus the C^{14} labelled organic acids appeared as peaks of radioactivity when C^{14} content and fraction number were compared (Figure 3). The identities of the separated acids was determined by descending paper chromatography as described in the following section.

The individual pool sizes of the organic acids present in the endosperm tissue were determined using a 20 gm tissue sample. The beans were sliced, killed, and extracted as previously described for the tissue feeding experiments and proportionately larger amounts of solvents and resins were used to separate the organic acid fraction. After gradient elution of this fraction, formic acid present in the tubes from the fraction collector, was removed by aeration at 50°C. The acids remaining in the tubes after complete removal of the formic acid were dissolved in 1 ml of distilled water and titrated against standard NaOH solution using 0.1% phenolphthalein as indicator. During the titration, the solution was stirred by means of CO_2-free air.

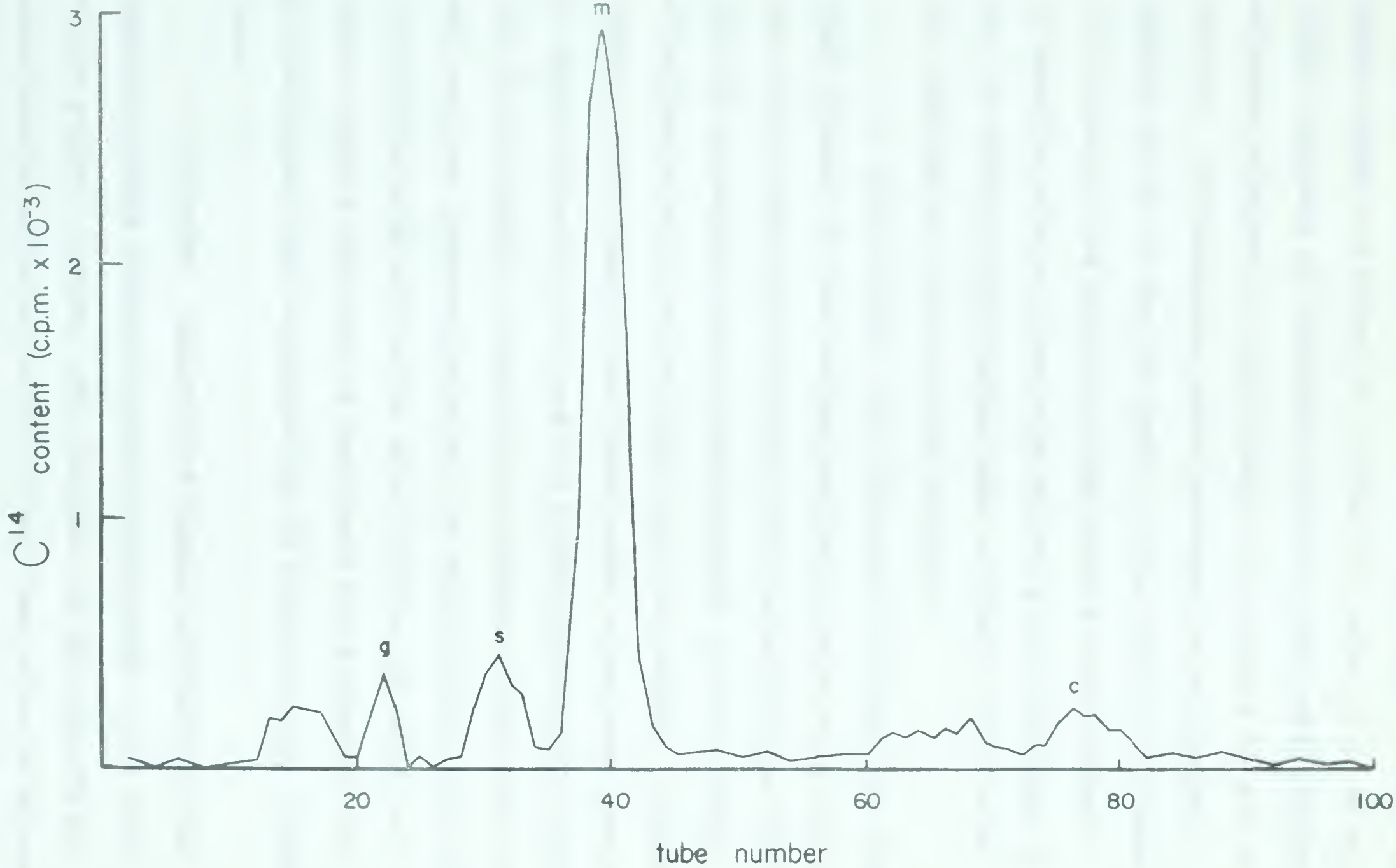
4) Separation of Labelled Compounds by Paper Chromatography:

a. Organic acids: After concentration of the isolated acids to a small volume, aliquots of the radioactive

FIGURE 3.

Gradient Elution of Labelled Organic Acids from Castor Bean Endosperm Tissues

Organic acids eluted as described in the text. Radioactive peaks correspond to: g glycollate, s succinate, m malate, and c citrate.


m
3
2
1
C^{14} content (c.p.m. x 10^{-3})
g
s
c
20
40
60
80
100
tube number

peaks were applied to a sheet of Whatman No. 1 chromatography paper and dried in a stream of warm air. In all cases, aliquots of authentic organic acids were applied as spots adjacent to the radioactive aliquots to act as markers. Chromatograms were developed in n-propanol: ammonia 60:40 v/v for 36 hours. After drying to remove all traces of the solvents, the markers were located by spraying with a 0.46% brom cresol purple solution in 6:1 v/v ethanol:formaldehyde made slightly alkaline by the addition of a dilute NaOH solution.

b. Amino acids: The individual radioactive amino acids present in the neutral and basic amino acid and in the acidic amino acid fractions were separated and identified by descending paper chromatography in the presence of authentic amino acids which acted as markers. Chromatograms of the neutral and basic amino acids were developed in phenol:water 8:3 v/v and the markers were later sprayed with 0.1% ninhydrin dissolved in acetone. The solvent system used for chromatography of the acidic amino acids was n-butanol:acetic acid:water 4:1:5 v/v/v. In order to achieve good separation of aspartate and glutamate, the chromatograms were developed for 48 hours at room temperature.

c. Sugars: Radioactive sugars and markers were separated using n-butanol:acetic acid:water 4:1:5 v/v/v as the solvent system. The location of the markers was determined by spraying with 0.5% w/v benzidine in ethanol:

glacial acetic acid: 40% w/v aqueous trichloracetic acid 8:1:1 v/v/v.

5) Measurement of Radioactivity Present in Plant Extracts and Carbon Dioxide:

The radioactive content of the fractions isolated by these procedures was assayed using a gas-flow counter (C110B Nuclear-Chicago) equipped with a mylar end window and an automatic sample changer. Samples to be counted were plated onto nickel planchets, spread with 95% ethanol and dried quickly in a warm air stream under infra-red light. In all cases, the counts were corrected for background. Radioactivity in respired CO_2 was assayed after conversion to $BaCO_3$ by addition of excess $Ba(OH)_2$. The $BaCO_3$ so formed was carefully filtered onto discs of glass-fibre filter paper and dried thoroughly at 100°C. The dried discs were then mounted onto nickel planchets and the radioactivity present was assayed using the gas-flow counter. In all cases the counts were corrected for self-absorption and background.

6) Detection of Labelled Compounds on Paper Chromatograms:

Radioactive areas present on the developed paper chromatograms were detected using a 4 Pi Actigraph (Nuclear Chicago R1000A). These areas were then eluted using distilled water by the method described by Stiller (1959). The radioactive eluates were concentrated to a small volume in a stream of warm air and aliquots assayed for C^{14} as described above.

RESULTS

STUDIES ON THE ALCOHOL DEHYDROGENASE SYSTEM OF CASTOR BEAN ENDOSPERM

1) Changes in Alcohol Dehydrogenase Activity During Germination:

Experiments with castor bean seeds (Cossins and Turner 1962), indicated that the endosperm tissue contained large amounts of alcohol dehydrogenase activity when the endogenous ethanol content of the tissues was decreasing. If this enzyme system is implicated in the pathway for ethanol metabolism, tissues capable of metabolizing ethanol should contain an active alcohol dehydrogenase system. In the present investigations with castor bean tissue, the work of Cossins and Turner (1962) has been extended to ascertain whether changes in alcohol dehydrogenase activity accompany the germination process.

Crude homogenates were prepared and assayed for alcohol dehydrogenase activity as described in the Materials and Methods section. Alcohol dehydrogenase activity was detected in these homogenates at all stages of germination (Table I). Furthermore, the activity of this enzyme system fluctuated considerably during the germination period examined. These fluctuations in enzyme activity were obvious if the activity was expressed on the basis of fresh weight, dry weight, or protein content of the homogenates (Table I). It should be noted, however, that changes also occurred in the fresh weight and protein content of the endosperm over the germination

TABLE I

Changes in the Alcohol Dehydrogenase Activity of Germinating

Castor Bean Seeds (Spectrophotometric Method)

Enzyme extraction and assay procedure as described in the Materials and Methods section. Alcohol dehydrogenase activity expressed as units of enzyme activity. One unit is defined as that amount of enzyme causing an increase in absorbance of 0.001/minute at 340 mμ.

Age of tissue (days)	Total units of alcohol dehydrogenase	Units/gm f wt	Units/gm dry wt	Units/mgm protein
1	6,200	1,240	1,650	14
2	10,200	2,040	2,650	24
3	7,800	1,560	2,030	22
4	6,700	1,340	1,980	17
5	7,200	1,440	2,360	11
6	5,500	2,310	4,620	17
7	8,900	1,770	4,430	25

period (Figure 4). It is evident that the alcohol dehydrogenase system present in homogenates of 2 and 7 day old endosperm had the highest specific enzyme activity.

In further studies on changes in the alcohol dehydrogenase activity during germination, the enzyme was assayed using the Thunberg tube technique (Table II). This method was employed in order to determine whether the data obtained would be comparable to those obtained from the spectrophotometric determinations. The results of duplicate determinations are shown in Table II. In agreement with the earlier determinations, the alcohol dehydrogenase activity fluctuated during the seven day germination period. The highest specific alcohol dehydrogenase activity was present in the 3 and 7 day old tissues. There was a marked decrease in the time necessary for decolourization of the blank tube as germination proceeded, especially in the oldest tissues. This might indicate a general increase in endogenous dehydrogenase activity as the tissue ages.

2) Properties of the Alcohol Dehydrogenase System in Castor Bean Endosperm:

Homogenates prepared in phosphate buffer were shown (Table I) to contain alcohol dehydrogenase activity. In further experiments, certain properties of this enzyme system were studied, including the effects of substrate and enzyme concentration, specificity, reversibility and inhibition. In these studies, crude extracts of endosperm tissue were employed. Although these preparations contained other enzyme

FIGURE 4.

Changes in Dry Weight and Protein Content of Castor Bean Endosperm Tissue During Germination

Dry weight (x—x) determined by drying 1 gm of slices at 100°C for 24 hours. Protein (o—o) determined as described in the Materials and Methods section.

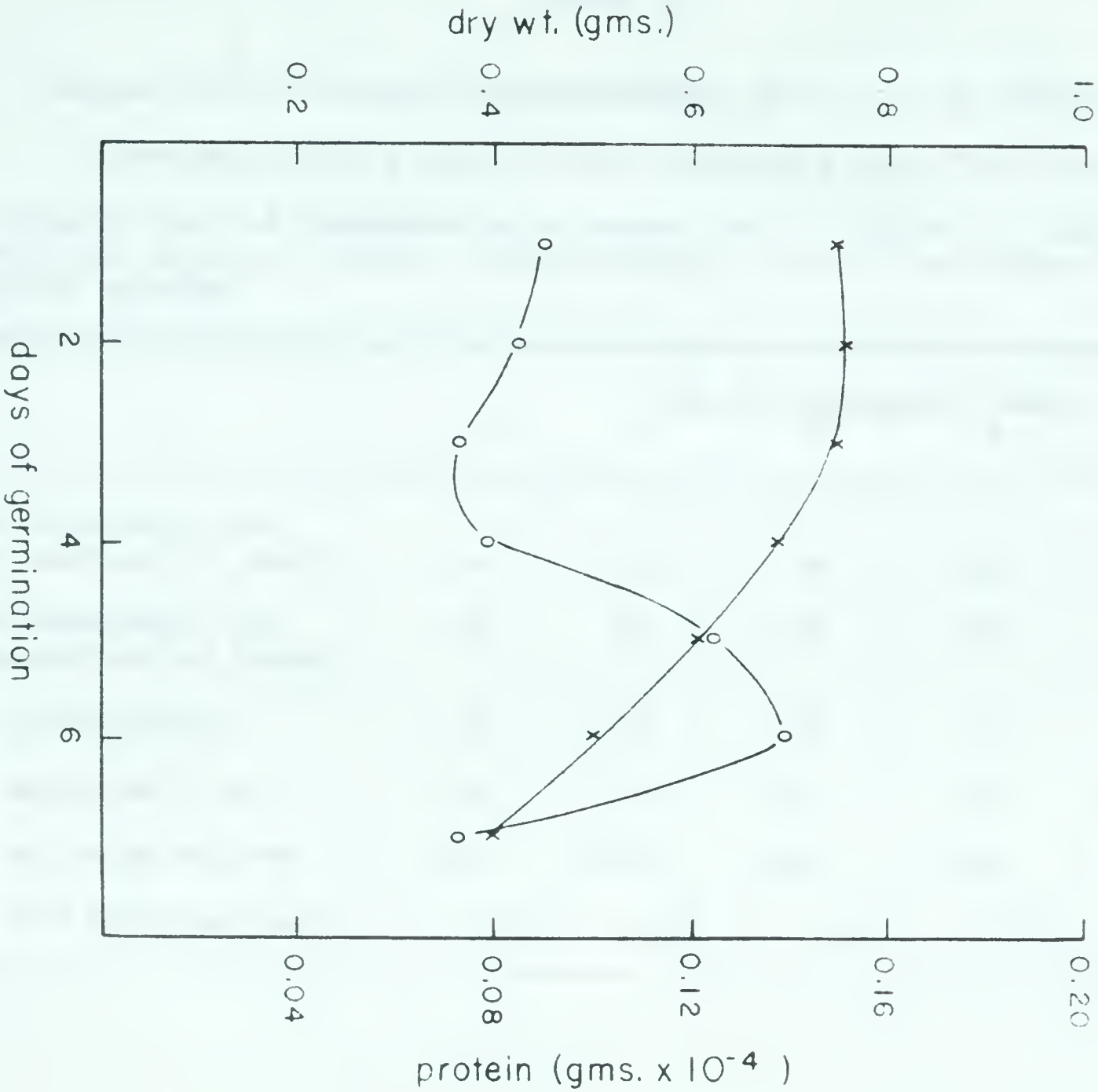

dry wt. (gms.)
0.2
0.4
0.6
0.8
1.0
days of germination
2
4
6
0.04
0.08
0.12
0.16
0.20
protein (gms. x 10^{-4})

TABLE II

Changes in the Alcohol Dehydrogenase Activity of Castor Bean Endosperm During Germination (Thunberg Tube Technique)

Preparation of homogenates as described in Table I. Assay procedure and definition of units of alcohol dehydrogenase activity as described in the Materials and Methods section

	Age of endosperm (days)					
	1	2	3	4	6	7
Minutes necessary for decolourization of sample	14	10	8	16	9	5
Minutes necessary for decolourization of blank	60	18	45	39	20	8
µl dye reduced/hour	14	19	25	12	23	36
enzyme units/gm f wt	86	114	147	74	162	216
enzyme units/gm dry wt	115	148	183	108	300	540
enzyme units/mgm protein	0.96	1.38	2.04	0.94	1.12	3.00

systems capable of reducing NAD, preliminary experiments indicated that NAD reduction was negligible at the high dilutions of enzyme used, compared to the extensive NAD reduction which was observed when ethanol was present.

a. Effects of substrate and enzyme concentration:

In experiments with the alcohol dehydrogenase system of pea cotyledons, Cossins (1961), Cossins and Turner (1963), it was demonstrated that enzyme activity was affected by adding various concentrations of ethanol. Similarly, in the present studies, increasing the concentration of ethanol up to 0.1 M increased the optical density change observed during the experimental period as well as the initial rate of the enzyme catalyzed reduction of NAD (Figure 5). In the presence of NADP there was little or no change in absorbance during the experimental period (Figure 5).

As is shown in Figure 6, the amount of NAD reduction was related to the amount of enzyme present. Increases in the optical density change were greatest in the experiments using the greatest amounts of enzyme. Furthermore, equilibrium was attained more readily when only small amounts of enzyme were present.

b. Specificity of alcohol dehydrogenase:

Data published on the properties of alcohol dehydrogenase systems from animals, micro-organisms and higher plants indicate that several aliphatic alcohols can serve as substrate for the enzyme (Bonnichsen and Brink 1955; Racker 1955; De Moss 1955; Cossins and Turner 1962). Experiments to deter-

FIGURE 5.

Effect of Substrate Concentration on NAD Reduction by Alcohol Dehydrogenase

Homogenate prepared from 5 day old castor bean endosperm tissue used for a spectrophotometric determination of alcohol dehydrogenase activity as described in Materials and Methods section. Sample cuvettes contained 500 µmoles phosphate buffer (pH 8.0), 0.1 ml homogenate (0.41 µgm protein). In addition, 150 µmoles ethanol and 50 µmoles NAD (●—●), 30 µmoles ethanol and 50 µmoles NAD (x—x), 15 µmoles ethanol and 50 µmoles NAD (o—o), and 30 umoles ethanol and 50 umoles NADP (▲—▲). Total volume 3 mls.

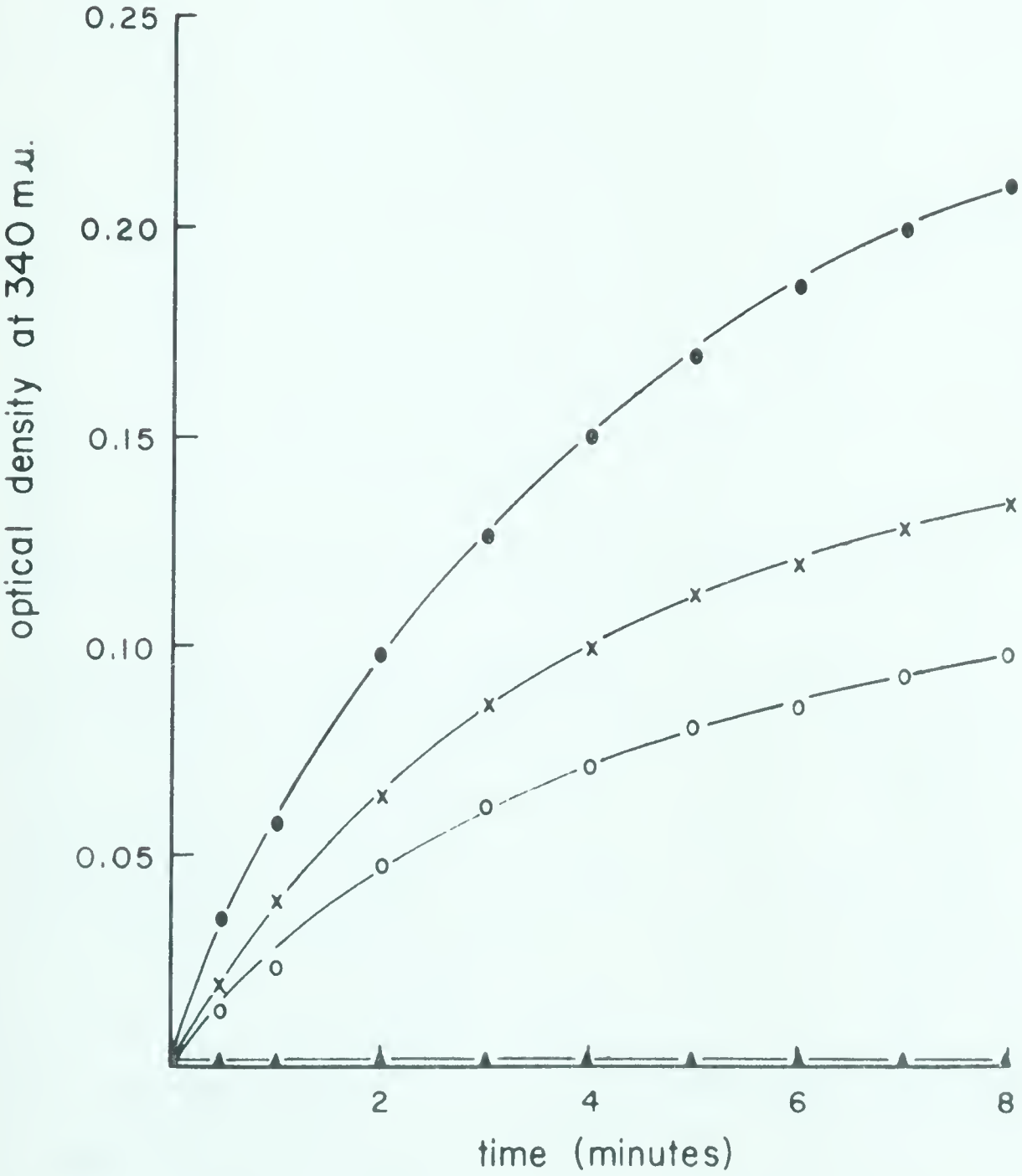
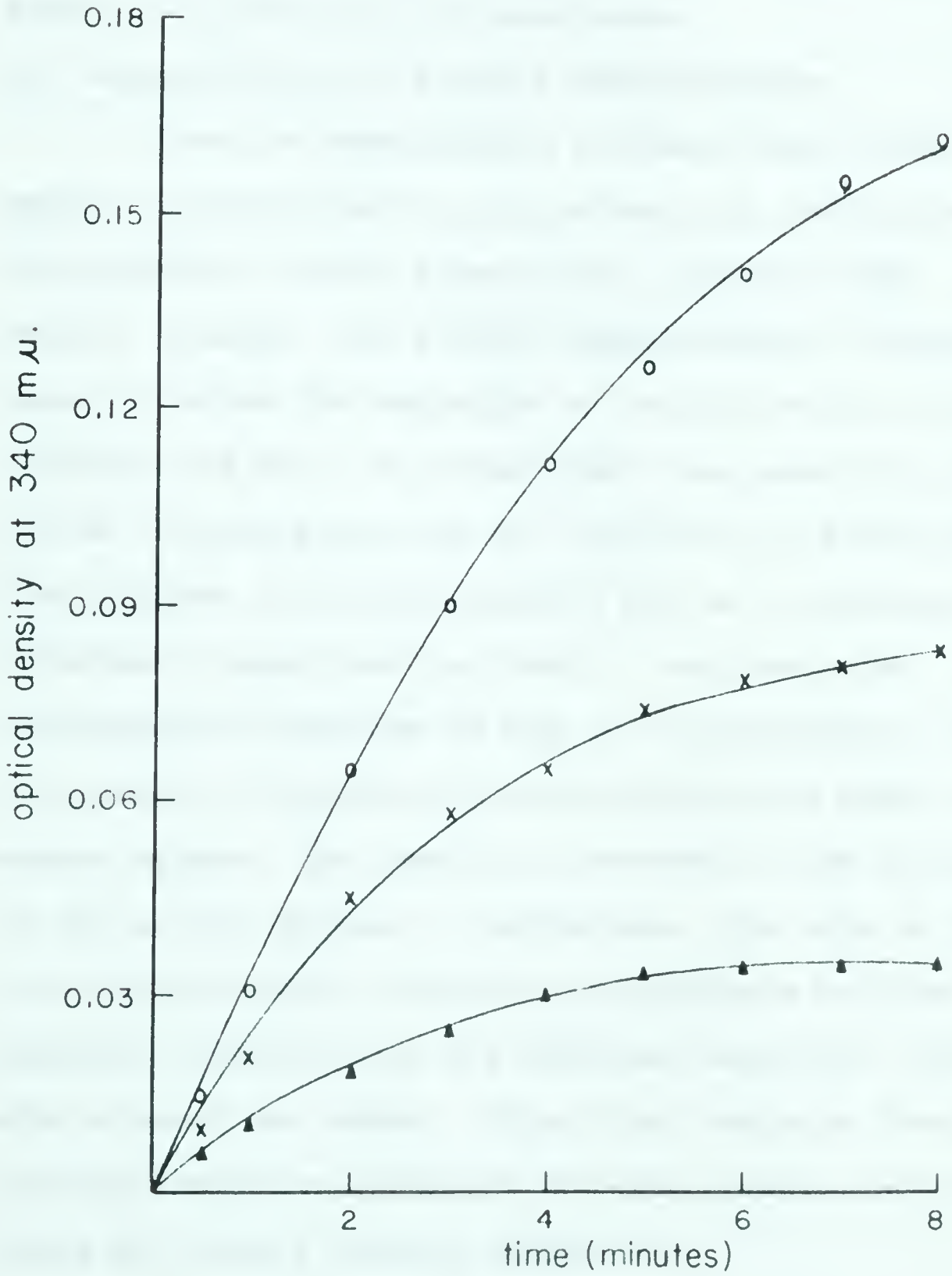

0.25
0.20
0.15
0.10
0.05
optical density at 340 mμ.
2
4
6
8
time (minutes)

FIGURE 6.

Effect of Enzyme Concentration on NAD Reduction

Homogenate prepared from 1 day old castor bean seeds as described in the Materials and Methods section. Alcohol dehydrogenase activity measured spectrophotometrically at 340 mμ. Sample cuvettes contained 50 μmoles NAD, 500 μmoles phosphate buffer (pH 8.0), 300 μmoles ethanol and the following additions of enzyme: o—o 0.2 ml homogenate (0.36 μgm protein); x—x 0.1 ml homogenate (0.18 μgm protein); ▲—▲ 0.05 ml homogenate (0.09 μgm protein). Total volume 3 mls.


0.18
0.15
0.12
0.09
0.06
0.03
optical density at 340 mμ.
2
4
6
8
time (minutes)

mine the degree of specificity of the alcohol dehydrogenase system of castor bean endosperm are summarized in Table III. Clearly, ethanol was the best substrate for the enzyme although additions of n-propanol and n-butanol resulted in considerable reduction of NAD. Reduction of NAD in the presence of methanol was only 4% of that observed when ethanol was added as the substrate.

c. Reversibility of alcohol dehydrogenase:

There is considerable evidence that alcohol dehydrogenase is implicated in the pathway for production of ethanol from pyruvate during anaerobiosis (Thomas 1958). In the present studies, the alcohol dehydrogenase system has been shown to cause the oxidation of ethanol with a concomitant reduction of NAD. To investigate the possibility that this system is reversible and can catalyze the reduction of acetaldehyde (utilizing reduced NAD as a co-enzyme) the experiment summarized in Figure 7 was conducted. After considerable reduction of NAD in the presence of 300 µmoles of ethanol, 30 µmoles of acetaldehyde were added to the enzyme system. An immediate decrease in the optical density at 340 mµ was obvious. Furthermore, the rate of decrease in optical density following acetaldehyde addition was considerably greater than the increase occurring initially when ethanol was added. This might indicate that the alcohol dehydrogenase system is not only freely reversible in vitro but favors ethanol formation.

TABLE III

The Specificity of the Alcohol Dehydrogenase Present in Castor Bean Endosperm

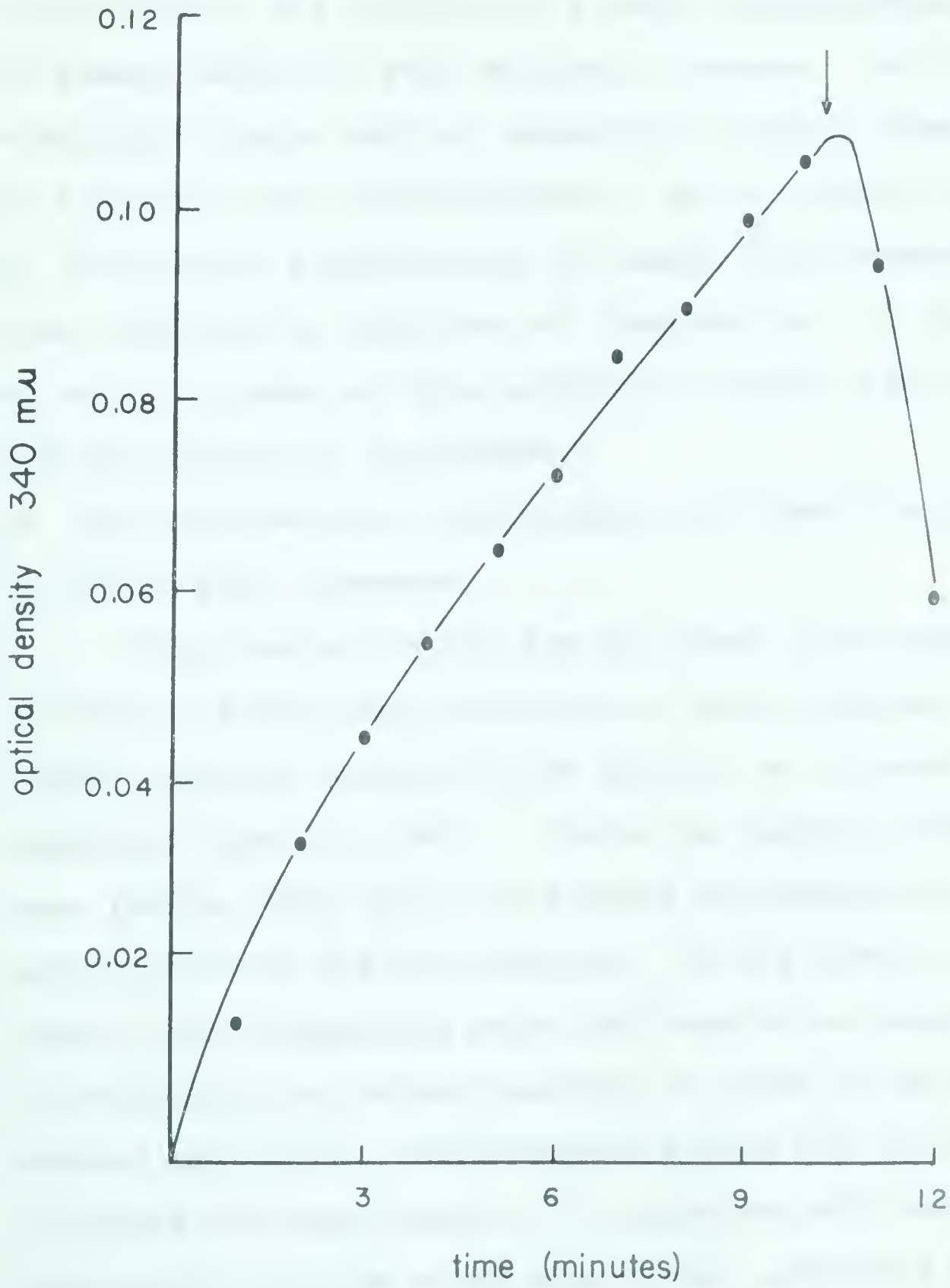
Homogenate prepared from 5 day old tissue as described in the Materials and Methods section. Alcohol dehydrogenase activity measured spectrophotometrically at 340 mμ, sample cuvette contained 300 μmoles of the alcohol, 50 μmoles NAD, 500 μmoles phosphate buffer (pH 8.0) and 0.1 ml of homogenate (containing 0.41 μgm protein) in a total volume of 3 ml.

Alcohol	Change in absorbance per minute per ml of enzyme	Relative alcohol dehydrogenase activity
Ethanol	0.060	100
n-Propanol	0.050	83
n-Butanol	0.045	75
Methanol	0.0025	4

FIGURE 7.

Reversibility of Alcohol Dehydrogenase Activity in Castor Bean Endosperm

Homogenate prepared from 5 day old castor bean endosperm tissue used in a spectrophotometric determination of enzyme activity as described in the Materials and Methods section. Sample cuvettes contained: 300 µmoles ethanol, 50 µmoles NAD, 500 µmoles phosphate buffer (pH 8.0) and 0.2 ml homogenate (containing 0.82 µgm protein) in a total volume of 3 ml. Arrow indicates the addition of 30 µmoles CH_3CHO.


0.12
0.10
0.08
0.06
0.04
0.02
optical density 340 mμ
3
6
9
12
time (minutes)

d. Inhibition of alcohol dehydrogenase activity:

Several reports in the literature (Cossins and Turner 1962; Rabin and Whitehead 1962; Stafford and Vennesland 1953) indicate that the activity of alcohol dehydrogenase systems of plants depend on free sulphydryl groups. For this reason, alkylating agents such as iodoacetate inhibit these systems at relatively low concentrations. As is evident from Figure 8, the alcohol dehydrogenase of castor bean endosperm was also inhibited by additions of iodoacetate. In the presence of only 0.1 μmole of this inhibitor, further reduction of NAD was completely suppressed.

3) The Intracellular Localization of Alcohol Dehydrogenase in Castor Bean Endosperm

Many enzyme systems are now known to be concentrated in certain subcellular particles in plant tissues. In this respect certain enzymes may be said to be intracellularly localized (Beevers, 1961). There are reports from pea tissues (Davies 1956) that the alcohol dehydrogenase system is associated with the mitochondria. In the present studies, castor bean homogenates were fractionated as described in the Materials and Methods section in order to determine whether the alcohol dehydrogenase system was intracellularly localized in these tissues. In agreement with earlier experiments, the low speed supernatant contained large amounts of alcohol dehydrogenase activity (Figure 9). Following centrifugation at high speed to remove the mitochondria, the supernatant fraction still contained

FIGURE 8.

Inhibition of Alcohol Dehydrogenase Activity by Iodoacetate

Homogenate prepared from 5 day old castor bean endosperm tissue used for spectrophotometric determination of alcohol dehydrogenase activity as described in the Materials and Methods section. Sample cuvettes contained: 50 µmoles NAD, 300 µmoles ethanol, 500 µmoles phosphate buffer (pH 8.0) and 0.2 ml homogenate (containing 0.82 µgm protein) in a total volume of 3 ml. Arrow indicates the addition of 0.1 µmole of iodoacetate. ●—● enzyme activity in absence of iodoacetate; o—o enzyme activity in presence of iodoacetate.

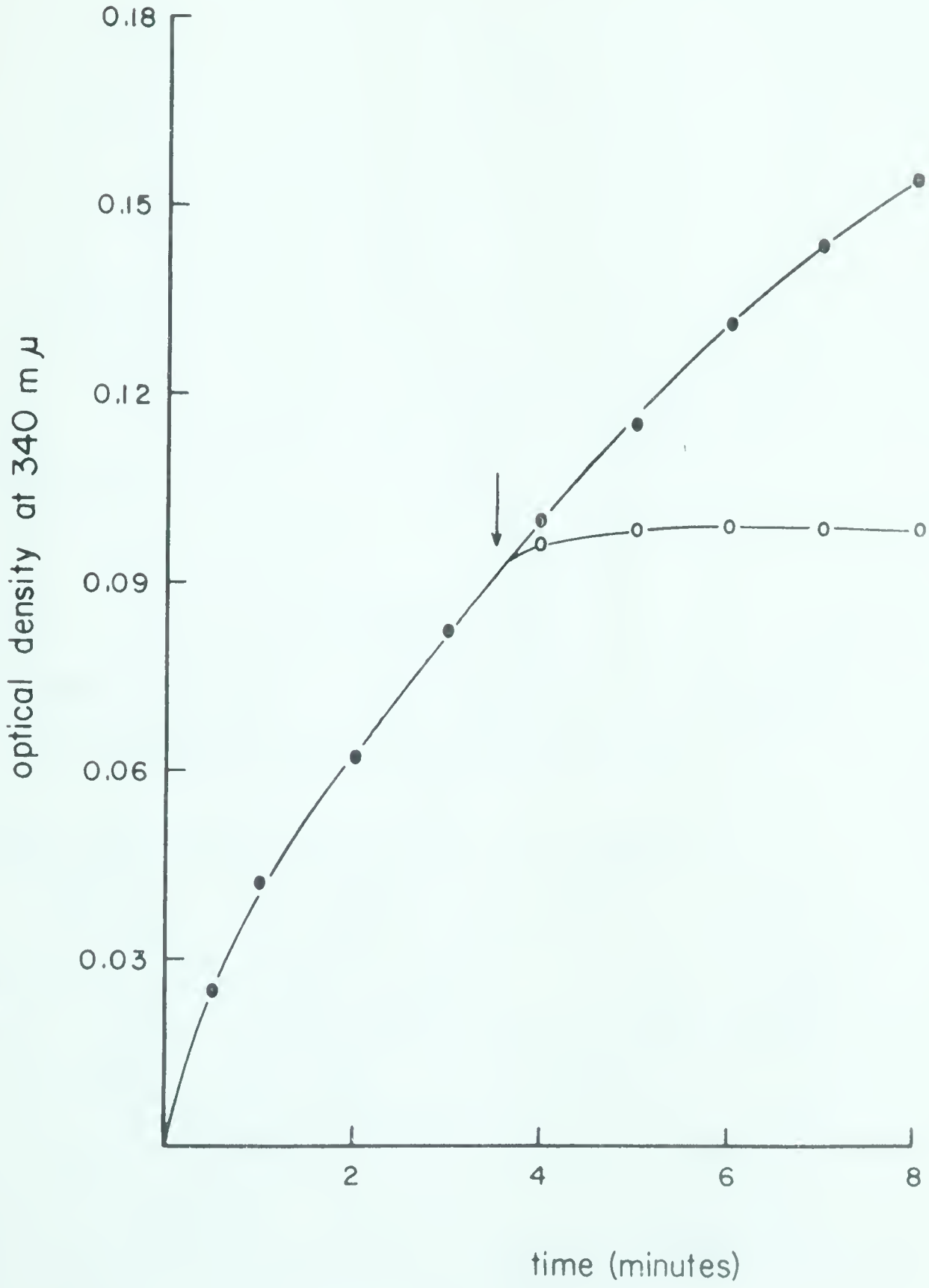
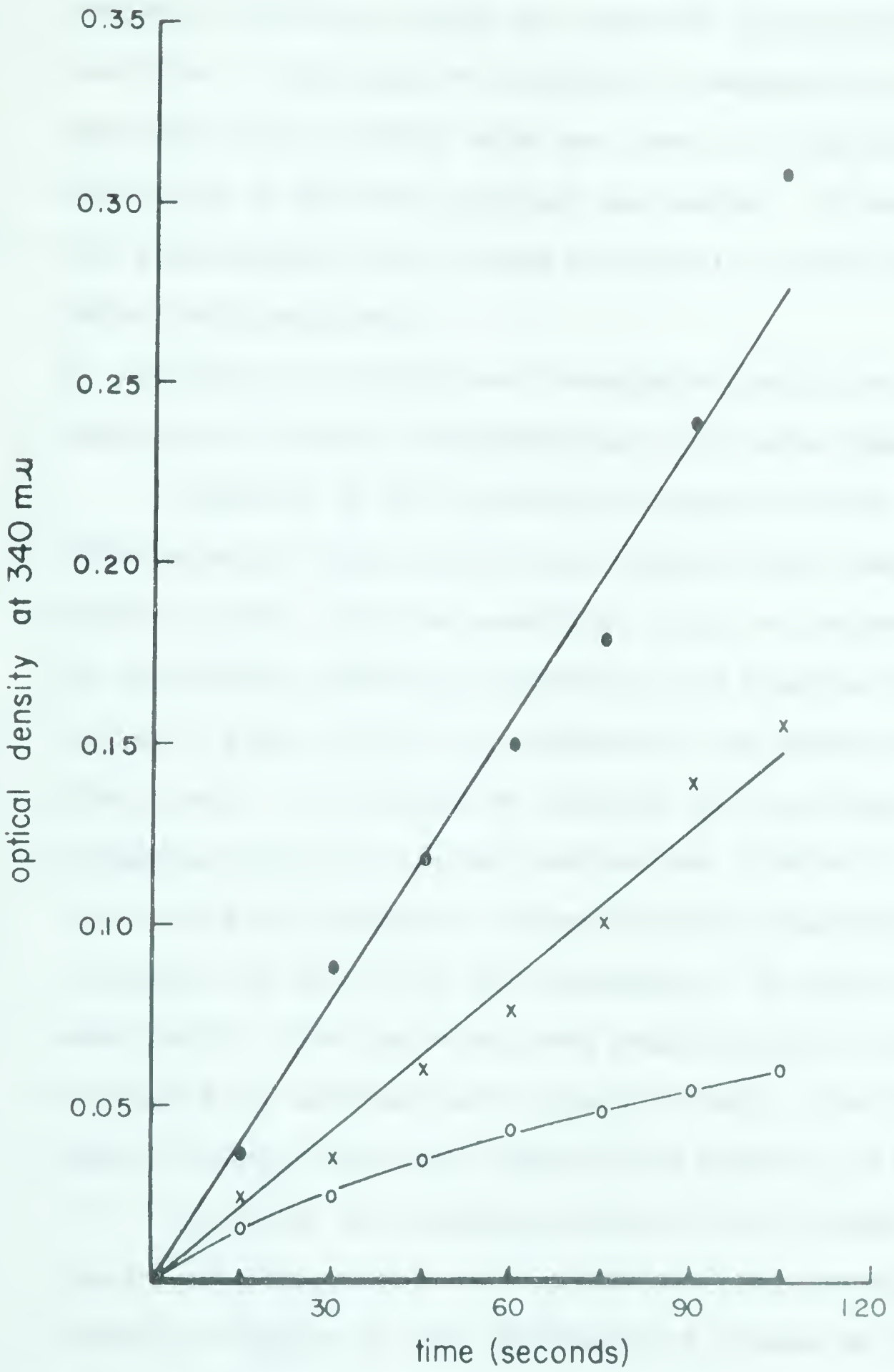

0.18
0.15
0.12
0.09
0.06
0.03
optical density at 340 mμ
2
4
6
8
time (minutes)

FIGURE 9.

Intracellular Localization of Alcohol Dehydrogenase Activity in Castor Bean Endosperm

Extracts of 5 day old castor bean endosperm tissue fractionated as described in Materials and Methods section. Alcohol dehydrogenase activity assayed spectrophotometrically at 340 mμ. Reaction mixtures (total volume 3 mls) contained: 500 μmoles phosphate buffer (pH 8.0) and 50 μmoles NAD. ●—● crude homogenate + 300 μmoles ethanol; x—x supernatant + 300 μmoles ethanol; o—o mitochondrial fraction + 120 μmoles malate; ▲—▲ mitochondrial fraction + 300 μmoles ethanol; △—△ mitochondrial fraction + 300 μmoles ethanol, + 30 μmoles cysteine.


0.35
0.30
0.25
0.20
0.15
0.10
0.05
optical density at 340 mμ
30
60
90
120
time (seconds)

large amounts of this enzyme. However, no alcohol dehydrogenase activity could be detected in the mitochondrial fraction. Additions of sulphydryl compounds such as cysteine (Davies 1956) did not result in any detectable reduction of NAD when ethanol was added. In contrast, the mitochondria were found to contain large amounts of malic dehydrogenase.

4) Effects of Aerobic and Anaerobic Conditions on the Activity of Alcohol Dehydrogenase in Castor Bean Endosperm:

Reports in the literature indicate that alcohol dehydrogenase activity of corn coleoptiles (Hagen and Flesher 1960) and rice seedlings (App and Meiss 1958) can be increased either by incubating the tissues with the enzyme's substrate or by subjecting the tissue to anaerobic conditions. In studies on changes in the alcohol dehydrogenase activity during germination (Table I) it was clear that the specific enzyme activity rose sharply to a maximum in the 3 day old seedlings. At this stage of germination, the radicles were emerging and it might be assumed from earlier work (Cossins 1961), that the endosperm tissues contained appreciable amounts of ethanol.

In order to determine whether the alcohol dehydrogenase activity might be related to endogenous ethanol content, samples of the tissues were placed in nitrogen or were aerated. Following these treatments, the alcohol dehydrogenase activity was determined. The results are

given in Table IV. Clearly, the tissues exposed to nitrogen contained much greater amounts of alcohol dehydrogenase activity than those which had been placed in moving air. Furthermore, the alcohol dehydrogenase extracted from the tissues in nitrogen had a considerably higher specific enzyme activity.

ETHANOL UTILIZATION DURING GERMINATION:

1) Sequence of Incorporation of Ethanol-C^{14}:

Ethanol has been shown to be metabolized by a variety of tissues, including 5 day-old castor bean endosperm (Cossins and Beevers 1963). The present studies were carried out to determine the sequence of reactions by which this metabolism might occur.

Experiments designed to study the utilization of ethanol involved a tissue slice technique to facilitate the penetration of the small amounts of substrate used in the experiments. Thin slices were prepared as described in the Materials and Methods section. At all times the sections were kept moist and in preliminary experiments viability of the tissue slices under experimental conditions was determined by following oxygen uptake using a Warburg respirometer. Figure 10 illustrates the oxygen uptake occurring when samples of the tissue slices were incubated for 10 hours. In both cases, the amount of tissue used was 0.5 gm and the volumes of liquid surrounding the slices were representative of those used in later experiments. The figure shows that a slight stimulation of oxygen uptake occurred over

TABLE IV

The Effects of Aerobic and Anaerobic Conditions on the Activity of Alcohol Dehydrogenase Present in Castor Bean Endosperm

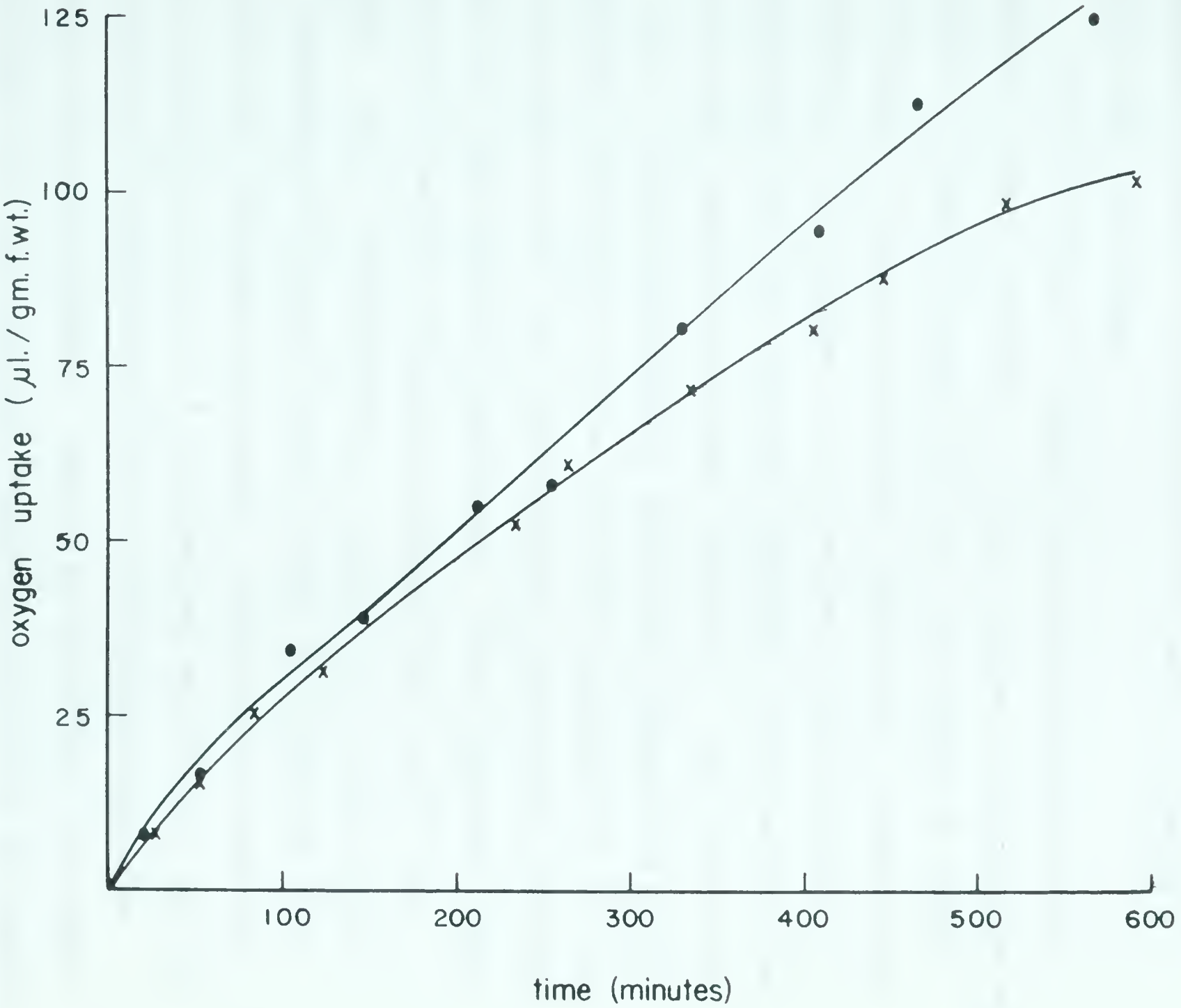
Testas removed from castor beans prior to treatment. Alcohol dehydrogenase activity assayed spectrophotometrically as described in the Materials and Methods section.

Treatment	Age of endosperm (days)	Total units	Units/gm f wt	Units/gm dry wt	Units/mgm protein
In moving air for 12 hours	6	3,750	1,500	3,000	9
In moving nitrogen for 12 hours	6	7,900	2,260	4,530	16
In moving air for 12 hours	7	17,000	2,100	5,250	28
In moving nitrogen for 12 hours	7	46,400	5,270	13,180	122

FIGURE 10.

Oxygen Uptake by Castor Bean Endosperm Tissue Slices

0.5 gm of 5 day old castor bean endosperm tissue slices incubated at 30°C for 10 hours. Oxygen uptake determined as described in the Materials and Methods section. Slices incubated in 0.2 ml distilled water (o—o), and 0.3 ml buffered medium (x—x).


125
100
75
50
25
oxygen uptake (µl./gm. f.wt.)
100
200
300
400
500
600
time (minutes)

the first 50 minutes of incubation. Following this, oxygen uptake continued linearly for the remaining experimental period. In the later experiments, the experimental feeding periods never exceeded 4 hours and the tissue slices were therefore assumed to be viable and to respire normally during this time period.

The metabolism of ethanol by slices of castor bean endosperm tissue prepared at various stages of germination was studied by feeding ethanol-1-C^{14} to 1 gm samples of tissue in Erlenmeyer flasks fitted with a centre well as described in Materials and Methods. It is evident from the total C^{14} incorporated that the 4 day-old endosperm tissue metabolized the greatest amounts of ethanol (Table V). The distribution of the C^{14} varied considerably during the germination period examined. Differences in the total C^{14} incorporated and the distribution of C^{14} into the fractions isolated were obvious over the 9 day period of germination. The 4 day-old tissue showed the greatest percentage incorporation of C^{14} into lipids. Up to the seventh day, the per cent of the total C^{14} incorporated into the organic acids declined and that into the CO_2 and sugars increased. Following the seventh day, a decline in total C^{14} incorporated and the per cent of the total C^{14} incorporated into all fractions except the organic acids was evident. The age of the tissue was therefore found to be an important factor in the extent and pattern of ethanol utilization. In further experiments, the metabolism of ethanol by 5 and 7 day-old endosperm tissues was examined

TABLE V

Utilization of Ethanol-1-C^{14} by Castor Bean Endosperm Tissue During Germination

1 gm of endosperm slices incubated 4 hours at 25°C with 0.4 μmoles of ethanol (containing 2 μc of C^{14}). Expressed as percentage of the total C^{14} incorporated into the various fractions.

Fraction	age of endosperm in days					
	1	3	4	6	7	9
Carbon dioxide	7	4	17	18	49	43
Lipids	9	30	38	35	10	2
Sugars	5	11	5	23	24	17
Organic acids	33	18	10	9	7	30
Amino acids	46	37	30	15	10	8
Total C^{14} incorporated (cpm)	9,780	12,190	25,080	10,440	10,230	10,040

in more detail in order to ascertain the metabolic events leading to the differences in ethanol utilization at these two stages of germination. At the later stage, the glyoxylate cycle has been shown to occur in the endosperm tissue (Canvin and Beevers 1961).

In order to determine the primary products of ethanol metabolism, tissues were allowed to utilize ethanol for very short periods of time. Table VI gives the results of a time-sequence study of ethanol-1-C^{14} metabolism. After only 60 seconds, ethanol carbon was incorporated into every fraction isolated. However, the rate of incorporation of ethanol-C^{14} into these fractions was rather slow for the first 3 minutes. After this lag period, the rate of incorporation of the radioactive substrate increased greatly. The total C^{14} content in each fraction continued to rise during the duration of the experiment with greatest increases occurring in the organic acid and amino acid fractions. In one minute the organic acid fraction contained the greatest amounts of the total C^{14} incorporated. The percentage of the total C^{14} incorporated into this fraction gradually declined and that present in the acidic amino acid fraction rose. This might indicate that the label was first incorporated into the organic acids and then spread into the acidic amino acids. Since the organic acids are commonly involved in intermediary metabolism and the acidic amino acids are known to be in equilibrium with them, this conclusion is clearly possible.

TABLE VI

Sequence of Incorporation of Ethanol-1-C^{14} in Seven-Day Old Castor Bean Endosperm Tissue

0.5 gm of endosperm slices incubated at 25°C with 1 µmole of ethanol containing 5 uc of C^{14} for time periods as indicated.

Fraction	1 minute		2 minutes		3 minutes		5 minutes	
	C^{14} (cpm)	% of inc. C^{14}	C^{14} (cpm)	% of inc. C^{14}	C^{14} (cpm)	% of inc. C^{14}	C^{14} (cpm)	% of inc. C^{14}
Lipids	280	14	310	12	780	18	2,200	13
Sugars	130	6	200	8	210	6	990	6
Organic acids	1,260	64	1,520	59	2,200	50	9,100	54
Neutral & basic amino acids	100	5	200	8	670	14	500	3
Acidic amino acids	200	11	350	13	540	12	4,200	24
Total C^{14} incorporated	1,970		2,580		4,400		16,990	

After 5 minutes of ethanol-1-C^{14} utilization, 67% of the C^{14} incorporated into the organic acids was recovered in malic acid. Glycollate, succinate, citrate and other acids contained 2%, 6% and 14% of the radioactivity in the organic acids respectively. In the other labelled fractions, however, the percentage of C^{14} incorporated remained low.

A comparison of the percentage incorporations of C^{14} into the various fractions after 5 minutes incubation (Table VI) with those after 4 hours (Table V), shows that an extended period of incubation results in a greater percentage of the total C^{14} incorporated entering CO_2 and the sugar fraction. Evidently ethanol metabolism has not reached a steady state after 5 minutes of incubation.

Results from a time-sequence study of longer duration are recorded in Table VII. Ethanol-2-C^{14} was used in this experiment. The percentage of the total C^{14} incorporated into the various fractions indicated that synthesis of sugars and neutral and basic amino acids might occur from the organic acids. A decrease in the percentage of the C^{14} entering the latter fraction was accompanied by increases in the percentage of C^{14} being incorporated into the sugars and neutral and basic amino acids.

A study of the incorporation of C^{14} into the organic acids from a labelled substrate in animal tissues can yield information regarding the sequence by which the substrate is incorporated. In these tissues an acid having the greatest

TABLE VII

Sequence of Incorporation of Ethanol-2-C^{14} by Seven-Day Old Castor Bean Endosperm Tissue

1 gm of slices incubated in air at 25°C in 6.7 µmoles of ethanol containing 10 uc of C^{14}. Time periods as indicated.

Fraction	5 min		15 min		30 min		60 min	
	C^{14} (cpm)	% of inc. C^{14}	C^{14} (cpm)	% of inc. C^{14}	C^{14} (cpm)	% of inc. C^{14}	C^{14} (cpm)	% of inc. C^{14}
Carbon dioxide	20	0	100	0	140	1	1,500	3
Lipids	930	13	2,800	11	5,100	12	9,600	16
Sugars	1,500	21	6,400	26	11,400	27	18,600	31
Organic acids	2,500	35	8,200	33	10,800	26	10,500	17
Neutral & basic amino acids	330	5	4,100	16	9,800	23	13,300	22
Acidic amino acids	1,800	26	3,500	14	4,700	11	6,400	11
Total C^{14} incorporated	7,080		25,100		41,940		59,900	

amounts of label and the highest specific activity can be assumed to be the initial product. Such a study with plant tissues is, however, complicated by the possibility that the organic acids may exist in distinct metabolic and storage "pools". If these two pools are in equilibrium to some extent, the C^{14} entering the turnover pool gradually spreads to the storage pool. Care must therefore be taken in interpreting data derived from feeding experiments with plant tissues.

In order to correctly interpret the results obtained from the fractionation of the organic acids, it was necessary to determine the quantity of each present in the tissue. A 20 gm tissue sample was prepared for titration as described in Materials and Methods. The quantities of the principal acids found in the 5 and 7 day-old castor bean endosperm tissues are presented in Table VIII. The acid content of the 7 day sample was considerably less than that of the 5 day sample, due mainly to decreases in the malate and citrate pools. Decreases in pool sizes of malate and citrate were partially compensated for by increases in succinic acid during this interval of germination. Differences in the size of the malate and citrate pools suggested that these acids exist in storage pools in this tissue. The decreases in malate and citrate might also indicate their utilization.

Whether such "compartmentation" of acids remote from the metabolic pool occurs can be determined from time-course

TABLE VIII

Pool Sizes of Organic Acids in Five and Seven Day Old Castor Bean Endosperm Tissue

Organic acid content of 20 gm sample of tissue determined as described in the Materials and Methods section

Organic acid	5 day old		7 day old	
	umoles acid per gm f wt	% of total ident. acids	umoles acid per gm f wt	% of total ident. acids
Glycollate	0.08	0	0.08	1
Succinate	0.21	2	2.10	25
Malate	7.85	77	5.50	66
Citrate	2.06	21	0.67	8
Total identified acids (umoles/gm f wt)	10.20		8.35	

experiments where incorporation of C^{14} into the individual organic acids can be studied (MacLennan, Beevers and Harley 1963). If a storage pool exists apart from the turnover pool, this spatial separation is destroyed when the tissues are extracted. After a long experimental time period, clearly the total C^{14} content of any particular organic acid might reflect its relative pool size rather than its importance in the metabolic sequence. During a feeding experiment using labelled substrate, C^{14} will quickly saturate the small metabolic pools giving them a high specific activity which rises quickly to a constant level. If the supply of labelled substrate becomes depleted during the experimental period, the specific activity of the metabolic pools will decline. If a storage of acid is in equilibrium with the metabolic pool, the specific activity of the total acid extracted will be low initially but will increase as C^{14} enters the storage pool. Table IX shows the changes in the specific activities of the labelled organic acids occurring during ethanol-2-C^{14} feeding. The total C^{14} incorporated and the specific activities of each organic acid increased during the experimental period at approximately equal rates. Of the acids examined, glycollate had the highest specific activity. The specific activities of malate and citrate were in all cases lower than that observed for glycollate. As was indicated earlier, this might result from a considerable dilution of C^{14} in the relatively large pools of malate and citrate in the tissues.

TABLE IX

Changes in the Specific Activities of Organic Acids in Seven-Day Old Castor Bean Endosperm Tissue During Ethanol-2-C^{14} Utilization

0.5 gm of endosperm slices fed 0.08 µmoles of ethanol containing 0.5 µc of C^{14}. Specific activity expressed as cpm/µmole of acid.

Organic acid	umoles/0.5 gm f wt	5 min		15 min		30 min	
		specific activity	ratio*	specific activity	ratio*	specific activity	ratio*
Glycollate	0.04	2,790	8.8	4,500	7.1	20,260	6.8
Succinate	1.05	317	1.0	634	1.0	2,990	1.0
Malate	2.75	400	1.3	1,430	2.3	4,390	1.5
Citrate	0.34	990	3.1	1,430	2.3	4,200	1.4

* Ratio calculated where succinate = 1

If ethanol is metabolized via the partial reactions of the glyoxylate cycle in the 7 day bean, clearly ethanol-1-C^{14} should label the respired CO_2 more extensively than ethanol-2-C^{14}. If carbohydrates are the major final products of the cycle, amounts of C^{14} entering CO_2 from the one position of ethanol should be similar to the amounts incorporated into sugars. On the other hand, the two position of ethanol should be a better precursor of sugars if the glyoxylate cycle is operating. Figure 11 illustrates the release of $C^{14}O_2$ from the individual carbon atoms of the ethanol molecule during a 2 hour feeding experiment in which equimolar amounts of ethanol-1-C^{14} and ethanol-2-C^{14} were fed to castor bean endosperm tissue. Ethanol-1-C^{14} was readily incorporated into the respired CO_2. A lag period of approximately 45 minutes was apparent before appreciable amounts of C^{14} were detected in the respired CO_2. Table X shows the distribution of C^{14} in the fractions derived from tissues incubated with ethanol-1-C^{14} and ethanol-2-C^{14}. The two position of ethanol was more readily incorporated into the sugars than the one position. The ratio of incorporation of C^{14} into sugars and CO_2 from the ethanol-1-C^{14} was 1:12. Data reported by Canvin and Beevers (1961) revealed that acetate-1-C^{14} feeding did not always result in the theoretical 1:1 ratio which should occur if sugar synthesis via the glyoxylate cycle is the only fate of acetate. These workers found that the amount of $C^{14}O_2$ released was always greater

FIGURE 11

Release of $C^{14}O_2$ from Ethanol-1-C^{14} and Ethanol-2-C^{14} by Castor Bean Endosperm Tissue

1 gm of 7 day-old castor bean endosperm slices incubated with equal specific activities (2 μc/32 μmole) of ethanol-1-C^{14} and ethanol-2-C^{14} at 25°C for 2 hours. C^{14} collected and assayed for radioactivity as described in the Materials and Methods section.

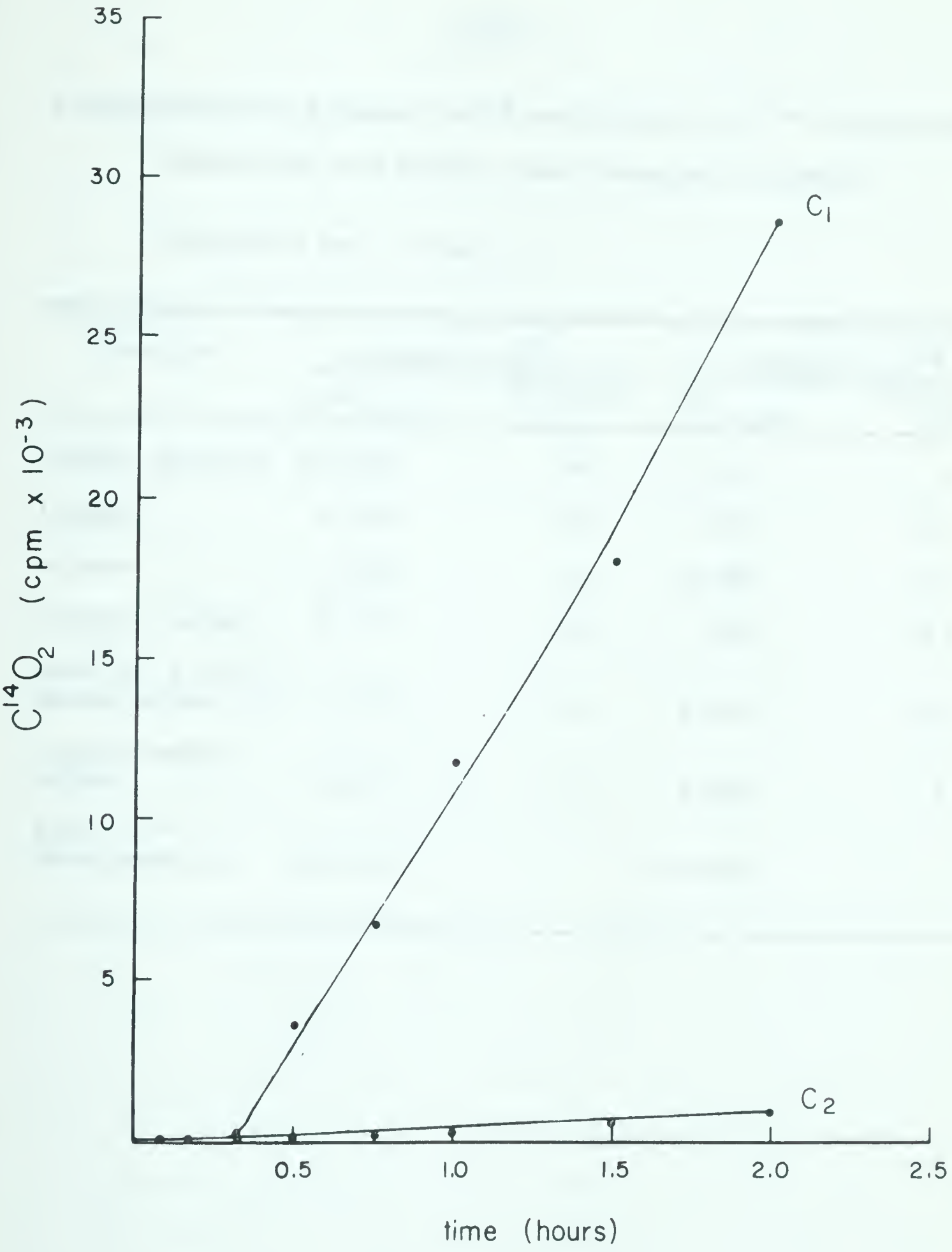

35
30
25
20
15
10
5
$C^{14}O_2$ (cpm x 10^{-3})
C_1
C_2
0.5
1.0
1.5
2.0
2.5
time (hours)

TABLE X

A Comparison of Ethanol-1-C^{14} and Ethanol-2-C^{14} Utilization

(Seven-Day Old Castor Bean Endosperm Tissue)

Procedure as in Figure 11.

Fraction	ethanol-1-C^{14}		ethanol-2-C^{14}	
	C^{14} (cpm)	% of inc. C^{14}	C^{14} (cpm)	% of inc. C^{14}
Carbon dioxide	28,900	49	1,100	4
Lipids	6,800	12	3,400	13
Sugars	2,100	4	8,800	35
Organic acids	8,400	14	3,200	13
Neutral & basic amino acids	6,600	11	6,700	26
Acidic amino acids	5,500	10	2,400	9
Total C^{14} incorporated	58,300		25,600	

than that incorporated into the sugar fraction in periods of incubation less than 2 hours. In this respect, the results of the ethanol-1-C^{14} feeding (Table X) resemble the results of acetate feeding (Canvin and Beevers 1961). The percentage of the total C^{14} incorporated into the lipid fraction was the same in the ethanol-1-C^{14} and the ethanol-2-C^{14} experiments. This suggests that the 2 carbons of ethanol are incorporated intact into the lipids. Greater percentages of the total C^{14} incorporated entered the neutral and basic amino acids when ethanol-2-C^{14} was fed.

2) The Effects of Glyoxylate and Oxaloacetate on Ethanol Metabolism:

When the glyoxylate cycle is operating, two-carbon units enter through the action of malate synthetase and the condensing enzyme. Since the former enzyme catalyzes the condensation of glyoxylate and acetyl CoA and the condensing enzyme catalyzes the condensation of oxaloacetate and acetyl CoA, additions of either glyoxylate or oxaloacetate should increase the total incorporation of acetyl CoA into the cycle. If ethanol is converted to acetyl CoA, the presence of either acid should increase the amounts of ethanol carbon being incorporated into the organic acids. In agreement with this suggestion, slices which were incubated with glyoxylate during ethanol-1-C^{14} feeding, incorporated twice as much ethanol as the control (Table XI). Greater percentages of the total radioactivity incorporated were found in

TABLE XI

The Effects of Glyoxylate and Oxaloacetate on the Utilization of Ethanol-2-C^{14} in Seven-Day Old Castor Bean Endosperm Tissue

0.5 gm of endosperm slices incubated with 500 umoles of phosphate buffer (pH 5.5) for 30 minutes with glyoxylate and oxaloacetate (10 µmoles) as indicated. Slices incubated for a further 30 minute period at 30°C with 1 µmole of ethanol containing 5 µc of C^{14}.

Fraction	ethanol-2-C^{14}		ethanol-2-C^{14} + glyoxylate		ethanol-2-C^{14} + oxaloacetate	
	C^{14} (cpm)	% of inc. C^{14}	C^{14} (cpm)	% of inc. C^{14}	C^{14} (cpm)	% of inc. C^{14}
Carbon dioxide	11,200	17	33,000	24	8,700	17
Lipids	10,700	16	10,900	8	9,900	19
Sugars						
Sucrose	14,000	20	41,700	31	13,000	26
Glucose	not detected		900	0	not detected	
Organic acids	16,000	23	30,500	22	10,000	20
Neutral & basic amino acids	12,600	18	14,300	11	6,000	12
Acidic amino acids	4,300	6	5,800	4	2,700	6
Total C^{14} incorporated	68,800		137,100		50,300	

CO_2 and sugars as a result of this treatment. The major component of this fraction was sucrose, in agreement with the data reported by Canvin and Beevers (1961) from acetate-C^{14} feeding experiments. Table XII shows that the major labelled component of the organic acid fraction was malate which was considerably increased over the control. Increases in the C^{14} content of the other identified acids were also obvious, particularly in citrate.

Additions of oxaloacetate, however, resulted in a 14% decrease in the total amounts of C^{14} incorporated without appreciably changing the percentage incorporated into any of the fractions. Analysis of the organic acid fraction (Table XII) showed that the C^{14} content of citric acid was not affected by oxaloacetate additions. Clearly, oxaloacetate did not give a 'sparker' effect as was evident when glyoxylate additions were made. Although this effect is difficult to explain adequately without further work, it is known that dicarboxylic acids are taken up by castor bean tissues at very low rates (Canvin and Beevers 1961). Alternatively oxaloacetate might have been rapidly metabolized by the tissues. Either of these alternatives would result in only small amounts of oxaloacetate being present to condense with acetyl CoA.

3) The Effects of Iodoacetate and Malonate on Ethanol Metabolism:

In studies on intermediary metabolism, considerable

TABLE XII

The Effects of Glyoxylate and Oxaloacetate on the Incorporation of Ethanol-2-C^{14} by Seven-Day Old Castor Bean Endosperm Tissue (Organic Acid Fraction)

Experimental procedure as in Table XI.

Organic acid	ethanol-2-C^{14}		ethanol-2-C^{14} + glyoxylate		ethanol-2-C^{14} + oxaloacetate	
	C^{14} (cpm)	% of C^{14} inc.	C^{14} (cpm)	% of C^{14} inc.	C^{14} (cpm)	% of C^{14} inc.
Glycollate	2,500	16	3,000	10	1,500	15
Succinate	2,800	18	4,000	13	1,500	15
Malate	4,900	31	14,000	46	4,700	47
Citrate	400	3	4,300	14	not detected	
Other acids	5,400	34	5,200	17	2,300	23
Total C^{14} incorporated into organic acids	16,000		30,500		10,000	

data concerning pathways and enzyme systems have been derived from experiments using metabolic inhibitors (James 1953a). In the present studies, iodoacetate and malonate, which are known to inhibit alcohol dehydrogenase and succinic dehydrogenase, were used in order to determine the importance of these enzymes in ethanol metabolism. In these experiments, the tissues were preincubated with the inhibitors for 30 minutes prior to the addition of ethanol-1-C^{14} (Table XIII). As has been shown previously (Figure 8), iodoacetate inhibited alcohol dehydrogenase activity *in vitro*. If ethanol is oxidized to acetaldehyde by this enzyme system, the total incorporation of ethanol carbon into all the fractions isolated should be markedly decreased in the presence of this inhibitor. As indicated in Table XIII, additions of iodoacetate to the slices resulted in a striking decrease in the amounts of ethanol carbon incorporated into the products.

Similarly additions of malonate decreased the amounts of ethanol incorporation by 45% as compared to the control. However, malonate treatment also changed the distribution of C^{14} within the fractions isolated. For example, the percentage of the total C^{14} incorporated into the organic acids and the acidic amino acids was considerably increased over the control. These results are consistent with the view that the pathway for ethanol metabolism involves succinic dehydrogenase activity. Malonate treatment also resulted in a marked decrease in the amounts of C^{14} entering the respired CO_2 but the

TABLE XIII

Effects of Iodoacetate and Malonate on the Utilization of Ethanol-1-C^{14} by Seven Day Old Castor Bean Endosperm Tissue

0.5 gm of tissue slices preincubated at 30°C for 30 min with 10 mmoles of the inhibitors and 50 µmoles of phosphate buffer (pH 5.5) as indicated. Slices incubated for a further 120 min at 30°C with 0.4 umoles of ethanol-1-C^{14} containing 2 µc of C^{14}.

Fraction	ethanol-1-C^{14}		ethanol-1-C^{14} + iodoacetate		ethanol-1-C^{14} + malonate	
	C^{14} (cpm)	% of inc. C^{14}	C^{14} (cpm)	% of inc. C^{14}	C^{14} (cpm)	% of inc. C^{14}
Carbon dioxide	21,800	57	560	11	3,400	20
Lipids	12,700	33	1,300	24	8,600	51
Sugars	1,200	3	870	16	1,000	6
Organic acids	1,700	4	910	17	1,800	11
Neutral & basic amino acids	720	2	1,000	19	650	4
Acidic amino acids	410	1	700	13	1,400	8
Total C^{14} incorporated	38,530		5,340		16,850	

incorporation of C^{14} into lipids was increased over the control. From these results it might be concluded that alcohol dehydrogenase and succinic dehydrogenase are important enzymes in the pathway for ethanol metabolism in this tissue.

4) The Role of Acetate in Ethanol Metabolism:

Experiments reported by Cossins and Beevers (1963) together with the ethanol-C^{14} feeding experiments described earlier in this thesis indicate that the organic acids are important products of ethanol metabolism. Entry of ethanol carbon into the organic acids possibly occurs via acetyl CoA. Ethanol carbon could be incorporated into the final products via acetyl CoA according to scheme (a) or (b) or might be metabolized by a pathway essentially different from that for acetate metabolism (scheme c).

(a) ethanol → acetaldehyde → acetate → acetyl CoA → products

(b) ethanol → acetaldehyde → acetyl CoA → products
↑
acetate

(c) ethanol → products

acetate → products

In the first series of reactions, ethanol is oxidized by an alcohol dehydrogenase system to form acetaldehyde which is oxidized further to acetate by an aldehyde dehydrogenase system. Acetate is then the substrate for an acetate activating enzyme, resulting in activation and condensation with coenzyme A to form acetyl CoA. Acetate is known to be

metabolized in castor bean endosperm tissue, presumably via conversion to acetyl CoA (Canvin and Beevers 1961). In scheme (b), acetaldehyde is converted directly into acetyl CoA and then to other products. Enzymes necessary for activation of acetaldehyde are known to occur in certain bacteria (Burton and Stadman 1953). The third possibility is that ethanol and acetate may be metabolized by entirely different pathways. If scheme (c) is the major pathway for ethanol incorporation, the addition of a large pool of acetate to a tissue utilizing ethanol should not affect the extent or nature of the products of ethanol metabolism. On the other hand, if ethanol is being metabolized via acetate or a compound in equilibrium with it, (scheme a or b) the addition of acetate would inhibit the incorporation of ethanol by an isotopic dilution effect. For example, carbon from ethanol mixing with the acetate pool would become considerably diluted and therefore smaller amounts of ethanol carbon would be incorporated into the final products. Radioactive carbon present in the ethanol molecule would therefore tend to be trapped in the increased acetate pool. Table XIV presents the results of an experiment designed to examine the effects of adding acetate to tissues metabolizing ethanol-C^{14}. The total C^{14} incorporated during the experimental period of 30 minutes was decreased by 72% when the tissue slices were incubated in the presence of 10 μmoles of acetate. This decrease in the total C^{14} was reflected in a

TABLE XIV

The Effect of Acetate on the Utilization of Ethanol-2-C^{14} by Seven-Day Old Castor Bean Endosperm Tissue

0.5 gm of endosperm slices preincubated for 30 minutes at 30°C with 50 μmoles of phosphate buffer (pH 5.5) and 10 μmoles of acetate as indicated. 6 μc of ethanol-2-C^{14} (4 μmoles) then added to each flask and the slices incubated at 30°C for a further 30 minutes.

Fraction	ethanol-2-C^{14}		ethanol-2-C^{14} + acetate	
	C^{14} (cpm)	% of inc. C^{14}	C^{14} (cpm)	% of inc. C^{14}
Carbon dioxide	1,800	7	820	10
Lipids	3,600	13	1,140	15
Sugars	5,800	21	1,500	19
Organic acids	5,900	21	3,100	40
Neutral & basic amino acids	6,700	24	770	10
Acidic amino acids	3,800	14	500	6
Total C^{14} incorporated	27,600		7,830	

general decrease in the amounts of C^{14} entering all the fractions isolated. This suggests that either acetate or acetyl CoA are important intermediates in ethanol metabolism.

If ethanol and acetate are both independently metabolized via conversion to acetyl CoA (scheme b), the addition of an ethanol pool might slow down the metabolism of acetate by competition for co-enzyme A. To test this possibility, acetate-C^{14} was fed to the tissues. If, however, ethanol is metabolized via acetate (scheme a), the addition of ethanol to the slices should not affect acetate-C^{14} incorporation. As shown in Table XV, additions of ethanol to the slices did not affect acetate-C^{14} incorporation. Scheme (a) therefore represents the major pathway by which ethanol carbon is incorporated by castor bean endosperm tissues. Separation of the labelled components present in the organic acid fraction (Table XVI), also indicated that the incorporation of acetate-C^{14} into these compounds was not appreciably altered by additions of ethanol.

As was shown previously, preincubation of the tissue slices with glyoxylate enhanced ethanol utilization. A similar stimulation of acetate-C^{14} metabolism was found to occur (Table XV) when additions of glyoxylate were made. However, the incorporation of C^{14} into malate was not significantly increased (Table XVI). In contrast, additions of glyoxylate to tissues metabolizing acetate-C^{14} were accompanied by increases in the amounts of C^{14} entering citrate.

TABLE XV

The Effects of Ethanol and Glyoxylate on the Utilization of Acetate-2-C^{14} in Seven Day Old Castor Bean Endosperm Tissue

0.5 gm samples of endosperm slices preincubated for 30 min at 30°C with 50 μmoles of phosphate buffer (pH 5.5); 10 μmoles of ethanol and 10 μmoles of glyoxylate as indicated. Following this, 4 μmoles of acetate containing 2 μc of C^{14} were added, and incubated at 30°C for a further 30 minutes.

Fraction	acetate-2-C^{14}		acetate-2-C^{14} + ethanol		acetate-2-C^{14} + glyoxylate	
	C^{14} (cpm)	% of inc. C^{14}	C^{14} (cpm)	% of inc. C^{14}	C^{14} (cpm)	% of inc. C^{14}
Carbon dioxide	710	4	660	4	840	4
Lipid	790	4	270	2	730	3
Sugars	1,200	7	1,300	7	3,000	14
Organic acids	11,900	66	10,900	63	13,500	60
Neutral & basic amino acids	2,400	13	2,900	17	2,450	11
Acidic amino acids	1,100	6	1,160	7	1,700	8
Total C^{14} incorporated	18,200		17,190		22,220	

TABLE XVI

The Effects of Ethanol and Glyoxylate on the Incorporation of Acetate-2-C^{14} by Seven Day Old Castor Bean Endosperm Tissue (Organic Acid Fraction)

Procedure as in Table XV.

Organic acid	acetate-2-C^{14}		acetate-2-C^{14} + ethanol		acetate-2-C^{14} +glyoxylate	
	C^{14}(cpm)	% of inc. C^{14}	C^{14}(cpm)	% of inc. C^{14}	C^{14}(cpm)	% of inc. C^{14}
Glycollate	1,200	11	900	8	1,200	9
Succinate	2,300	19	3,400	31	2,100	16
Malate	2,900	24	3,300	30	3,000	22
Citrate	2,600	22	2,600	24	5,300	39
Other acids	2,900	24	700	7	1,900	14
Total C^{14} incorporated into organic acids	11,900		10,900		13,500	

Isotopic dilution studies of ethanol and acetate metabolism (see above) have suggested that the two-carbon skeleton of ethanol is oxidized to acetate possibly via conversion to acetaldehyde. If such a reaction sequence is the major pathway for ethanol oxidation, the products of ethanol and acetate metabolism should be similar. When the fates of these two substrates were compared experimentally, using 7 day endosperm slices, certain differences in their patterns of incorporation were apparent (Table XVII). Although ethanol-C^{14} and acetate-C^{14} solutions of equal specific activities were fed to the tissue slices, the incorporation of acetate carbon was approximately seven times greater than that observed when ethanol-C^{14} was supplied. This difference in the rates of utilization of the compounds might be explainable if losses of ethanol-C^{14} occurred due to its volatility. Alternatively, if the conversion of ethanol to acetaldehyde is slow under the experimental conditions used, this step may limit the overall conversion of ethanol into the products. Greater percentages of the acetate carbon utilized were incorporated into sugars. Large amounts of ethanol carbon were incorporated into CO_2. The ratio of C^{14} incorporated into sugars to that incorporated into CO_2 was 2:3 from acetate-1-C^{14} and 1:19 from ethanol-1-C^{14}. Acetate was also a better precursor for amino acids, especially the neutral and basic amino acids, than was ethanol. Ethanol carbon was incorporated into the lipid fraction to a greater

TABLE XVII

A Comparison of Ethanol-1-C^{14} and Acetate-1-C^{14} Utilization in Seven-Day Old Castor Bean Endosperm Tissue

0.5 gm of endosperm slices preincubated at 30°C for 30 min in 50 µmoles of phosphate buffer (pH 5.5). Additions of 4 µmoles of ethanol and acetate (2 µc of C^{14}) were made and incubation continued at 30°C for a further 120 minutes.

Fraction	acetate-1-C^{14}		ethanol-1-C^{14}	
	C^{14} (cpm)	% of inc. C^{14}	C^{14} (cpm)	% of inc. C^{14}
Carbon dioxide	131,000	29	21,800	57
Lipids	12,300	3	12,700	33
Sugars	82,900	18	1,200	3
Organic acids	99,500	22	1,700	4
Neutral & basic amino acids	71,000	16	720	2
Acidic amino acids	55,100	12	410	1
Total C^{14} incorporated	451,800		38,530	

extent than occurred in the acetate feeding.

During the earlier stages of germination (5 day old tissues) the products of ethanol metabolism were different from those obtained from ethanol or acetate metabolism in 7 day tissues. The lipid-forming tendency of ethanol was more pronounced during the early stages of germination (Table V). A comparison of ethanol and acetate metabolism in 5 day old castor bean endosperm tissue was therefore made to determine whether differences in patterns of ethanol and acetate metabolism existed at this age. The results of this study are presented in Table XVIII. The striking differences in ethanol and acetate metabolism at this stage of germination suggest they may be utilized by essentially different pathways. Again, equimolar amounts of both ethanol and acetate were fed to the tissue samples. The specific activities of the ethanol and acetate solutions, however, were different, being 1.5 μc/umole and 0.25 μc/umole respectively. Acetate was incorporated twice as quickly as ethanol. Fifty seven per cent of the ethanol-2-C^{14} incorporated was present in the organic acid fraction. The corresponding figure for the acetate experiment was only 27%. Differences in the amounts as well as the identities of the sugars formed were also apparent. Alanine was the major neutral and basic amino acid formed from both substrates but ethanol was a better precursor for glutamic acid. When specific activities of the organic acids are compared (Table

TABLE XVIII

A Comparison of Ethanol-2-C^{14} and Acetate-2-C^{14} Utilization by Five-day Old Castor Bean Endosperm Tissue

0.5 gm of tissue slices incubated in 50 μmoles of phosphate buffer (pH 5.5) and 4 μmoles of ethanol containing 6 μc of C^{14} and 4 μmoles of acetate containing 1 μc of C^{14} for 120 minutes at 30°C.

Fraction	ethanol-2-C^{14}		acetate-2-C^{14}	
	C^{14} (cpm)	% of inc. C^{14}	C^{14} (cpm)	% of inc. C^{14}
Carbon dioxide	200	0	900	2
Lipids	2,100	2	1,100	2
Sugars				
Glucose	6,100	5	not detected	
Fructose	3,000	3	not detected	
Sucrose	not detected		15,700	29
Organic acids				
Glycollate	1,900	2	1,300	2
Succinate	7,100	6	1,500	3
Malate	34,800	31	5,100	10
Citrate	12,300	11	2,400	4
Others	7,400	7	4,400	8
Neutral & basic amino acids				
Alanine	22,600	20	14,200	27
Asparagine	not detected		1,100	2
Acidic amino acids				
Glutamate	10,900	10	1,000	2
Aspartate	3,400	3	4,700	9
Total C^{14} incorporated	111,800		53,400	

XIX), differences between ethanol and acetate metabolism are more apparent. It is difficult to compare the values of the specific activities directly, since a higher percentage of ethanol-C^{14} entered this fraction than occurred during acetate-C^{14} feeding. However, the ratios of the specific activities of the acids separated affords a more direct comparison. The ratios of the specific activities of malate : glycollate : succinate : citrate were 1:54:73:14 for ethanol, and 1:25:10:2 for acetate. Ethanol was apparently more readily incorporated into succinate. Variations in the labelling pattern of the organic acids might be due to a more rapid metabolism of acetate than ethanol. As was pointed out earlier, in 7 day-old castor bean endosperm, ethanol metabolism resembles acetate metabolism more than it does in 5 day-old tissues. However at both stages of germination, acetate was metabolized at a much faster rate than ethanol. To determine whether the differences in the specific activities of the various acids in the 5 day tissues were due to differences in metabolic pathways or to the more extensive metabolism of acetate at that time, specific activities of acids from the 7 day endosperm were compared (Table XX). At this stage of germination, the specific activities were found to be more similar.

5) Ethanol Metabolism in Corn Root Tips:

In order to determine how ethanol is metabolized in a tissue known to contain an active TCA cycle (Harley and

TABLE XIX

Specific Activities of Organic Acids Following Utilization of Ethanol-2-C^{14} for 120 Minutes (5 Day-Old Endosperm Tissues).

Procedure as in Table XVIII. Specific activity expressed as cpm/umole acid.

Organic acid	umoles acid per 0.5 gm f wt	ethanol-2-C^{14}		acetate-2-C^{14}	
		C^{14} (cpm)	specific activity	C^{14} (cpm)	specific activity
Glycollate	0.04	1,900	47,500	1,300	32,500
Succinate	0.11	7,100	64,600	1,500	13,600
Malate	3.93	34,800	8,850	5,100	1,300
Citrate	1.03	12,300	11,940	2,400	2,330
Total C^{14} incorporated into acids		55,800		10,300	

TABLE XX

Specific Activities of Organic Acids Following Utilization of Ethanol-2-C^{14} and Acetate-2-C^{14} (Seven Day-Old Endosperm Tissues)

0.5 gm of tissue slices incubated at 30°C for 30 minutes in 50 μmoles of phosphate buffer (pH 5.5). 4 μmoles of ethanol (containing 6 μc of C^{14}) and 4 μmoles of acetate (containing 1 μc of C^{14}) were then added to the flasks and incubation continued for 30 minutes. Specific activity expressed as cpm/μmole acid.

Organic acid	μmoles acid /0.5 gm f wt	acetate-2-C^{14} specific activity	acetate-2-C^{14} ratio*	ethanol-2-C^{14} specific activity	ethanol-2-C^{14} ratio*
Glycollate	0.04	17,450	8.0	3,550	5.3
Succinate	1.05	2,190	1.0	670	1.0
Malate	2.75	1,060	0.5	950	1.4
Citrate	0.34	7,650	3.5	1,180	1.2

* Ratio calculated where succinate = 1.

Beevers 1963), ethanol-2-C^{14} and acetate-2-C^{14} were fed to 3 day-old corn root tips. Ethanol was found to be a better precursor for lipids and alanine than was acetate (Table XXI). In contrast to the experiments with castor bean, greater percentages of the labelled substrate entered the respired CO_2 and considerably less sugar synthesis occurred. However, results obtained recently by Cameron and Cossins (unpublished data) using pea cotyledon tissues which also contain an active TCA cycle, indicate that ethanol and acetate are metabolized by similar pathways.

It appears that ethanol is predominantly metabolized by the glyoxylate cycle in 7 day old castor bean endosperm tissues and mainly by way of the TCA cycle in excised corn root tips. Lipid formation from ethanol was, however, a consistent feature of ethanol metabolism in all tissues examined.

TABLE XXI

A Comparison of Ethanol-2-C^{14} and Acetate-2-C^{14} Utilization by Three Day Old Corn Root Tips

0.5 gm of tissue incubated at 30° C for 220 minutes in 70 µmoles of phosphate buffer (pH 5.6) and 0.4 µmoles of ethanol (containing 2 µc of C^{14}); and in 0.4 µmoles of acetate (containing 2 µc of C^{14}) as indicated.

Fraction	acetate-2-C^{14}		ethanol-2-C^{14}	
	C^{14} (cpm)	% of C^{14} inc.	C^{14} (cpm)	% of C^{14} inc.
Carbon dioxide	99,000	17	2,300	13
Lipids	6,700	1	1,500	8
Sugars	7,600	1	440	2
Organic acids	432,000	76	8,200	44
Amino acids				
Alanine	6,800	1	3,800	20
Glycine & asparagine	5,200	1	not detected	
Glutamic	15,600	3	2,300	13
Aspartic	1,400	1	not detected	
Total C^{14} incorporated	574,300		18,540	

DISCUSSION

A limited explanation of certain observations has already been presented in the Results section. In the present section it is proposed to discuss the implications of the experimental data in more detail.

THE ROLE OF ALCOHOL DEHYDROGENASE IN ETHANOL METABOLISM:

The enzyme alcohol dehydrogenase is of widespread occurrence in higher plants. Davison (1949) found this system in 69 of 93 species of plant seeds examined. Besides being widely distributed, this enzyme is known to be involved in the formation of ethanol during anaerobic respiration (Fruton and Simmonds 1960). As the reaction is freely reversible *in vitro*, alcohol dehydrogenase can also cause the oxidation of ethanol to acetaldehyde. For example, recent work with animal tissues has indicated that the oxidation of ethanol to acetaldehyde *in vivo* occurs via an alcohol dehydrogenase system (Kinard *et al.* 1956 and Lundquist *et al.* 1963). If this enzyme system plays a major role in ethanol metabolism *in vivo*, it is clear that tissues such as castor bean endosperm tissue, known to metabolize ethanol (Cossins and Beevers 1963), might be expected to contain an active alcohol dehydrogenase system. This dehydrogenase system was in fact, present in homogenates of castor bean endosperm tissue throughout the nine day period of germination examined (Tables I and II). This dehydrogenase could therefore play

an important role during the utilization of ethanol by this tissue during germination. Results of ethanol-C^{14} feeding experiments (Table V), indicate that ethanol is readily utilized by the castor bean endosperm tissue at all stages of germination. Both the alcohol dehydrogenase activity and the ethanol-C^{14} incorporated into the tissue fluctuated during germination. However it appears that at no stage of germination was the metabolism of ethanol limited by the amounts of alcohol dehydrogenase present in the tissues. As is evident from the data presented in Tables I and V, maximal incorporation of ethanol carbon occurred when the tissues were 4 days old. At this stage of germination, the tissues contained considerably less alcohol dehydrogenase activity than was detected earlier in the germination period. Similarly in the seven day-old tissues, although the specific alcohol dehydrogenase activity was maximal, the amounts of ethanol-C^{14} incorporated were decreased.

The effects of anaerobic conditions on the amounts of alcohol dehydrogenase activity present in rice coleoptiles (App and Meiss 1958) and in the scutellum and embryonic axis of corn seedlings (Hageman and Flesher 1960) have been reported. In these tissues, alcohol dehydrogenase activity was found to be influenced by the O_2 supply. Enzyme activity decreased when the tissues were placed in aerobic conditions and increased during periods of anaerobiosis. These workers reported that this increased alcohol dehydrogenase activity could be induced

in rice coleoptiles by incubation with ethanol and in the corn tissues by treatment with acetaldehyde. In the present studies, alcohol dehydrogenase activity in the castor bean endosperm tissue also was found to be influenced by subjecting the tissues to an anaerobic environment (Table IV). Incubation in N_2 for a twelve hour period resulted in a striking increase in alcohol dehydrogenase activity as compared to the controls. Such an increase in enzyme activity could be due either to an activation of the alcohol dehydrogenase already present in the tissues or to a stimulation of enzyme synthesis by the anaerobic conditions. Further work is, however, necessary before definite statements can be made regarding the effects of anaerobic conditions on alcohol dehydrogenase activity.

Davies (1956) has reported that the alcohol dehydrogenase system of pea epicotyls is associated with the mitochondria. However, in the present work with castor bean tissues this enzyme was found to be entirely concentrated in the cytoplasmic fraction (Figure 9). No alcohol dehydrogenase activity could be detected in the mitochondrial fraction. In contrast, this fraction was found to contain an active malic dehydrogenase system in agreement with data published by other workers (Beevers 1961).

Perhaps the strongest argument for the involvement of alcohol dehydrogenase in ethanol metabolism in castor bean endosperm tissues is derived from the experiments in which

metabolic inhibitors were used. In agreement with previous reports from a variety of tissues (Cossins and Turner 1962, Stafford and Vennesland 1953 and Rabin and Whitehead 1962), the activity of alcohol dehydrogenase extracted from castor bean endosperm tissue was found to be completely inhibited by additions of iodoacetate (Figure 8). Furthermore, iodoacetate greatly affected the incorporation of ethanol-C^{14} into tissue slices (Table XIII). The incorporation of the label into all the fractions isolated was severely depressed, indicating that the iodoacetate inhibits ethanol metabolism. Clearly, this effect is consistent with the operation of an alcohol dehydrogenase system during ethanol metabolism. Similar conclusions have been made from the effects of inhibitors on ethanol metabolism in animal tissues (Kinard et al. 1956 and Lundquist et al. 1963).

THE ROLE OF THE GLYOXYLATE CYCLE IN ETHANOL METABOLISM:

Canvin and Beevers (1961) have presented extensive evidence for the operation of the glyoxylate cycle in castor bean endosperm tissue. This pathway allows for the synthesis of sugars from the two carbon units derived from fatty acid oxidation. The glyoxylate cycle, which is essentially a bypass of the TCA cycle, involves the net synthesis of organic acids (Scheme i). Results from ethanol-C^{14} incorporation studies have indicated that a large proportion of the ethanol carbon enters the organic acid pools. Furthermore as the incubation period with ethanol-C^{14} was decreased, greater

percentages of the total C^{14} incorporated were present in the organic acids (Table VI). Similar results have been obtained from acetate-C^{14} feeding in this tissue (Canvin and Beevers 1961). These data therefore indicate that the organic acids are primary products of ethanol and acetate metabolism in these tissues. Thus in the initial stages of ethanol metabolism, ethanol carbon is incorporated into the acids of the glyoxylate cycle (page 48). The large incorporation of C^{14} into malic acid after 5 minutes incubation with ethanol-C^{14} suggests that this acid is one of the first components of the cycle to become labelled. Clearly, this is consistent with malate synthetase activity occurring during ethanol metabolism. Further evidence for the role of malate synthetase in ethanol metabolism was obtained from experiments using glyoxylate (Table XII). When tissue slices were incubated with glyoxylate prior to ethanol-1-C^{14} feeding, the incorporation of the C^{14} was accompanied by large increases in the amounts of C^{14} entering sucrose and malic acid. These results give further evidence that the malate synthetase reaction is occurring during ethanol metabolism.

Glycollate-C^{14} was isolated from castor bean slices after incubation with acetate-C^{14} (Canvin and Beevers 1961). The presence of this acid was considered by these workers to be "strong presumptive evidence" for glyoxylate formation. The present work has indicated that glycollic acid, although present in small amounts (Table VIII), has a very high specific

activity (Tables IX and XX) following ethanol-C^{14} feeding. The action of glyoxylic acid reductase would produce glycollate-C^{14} from glyoxylate-C^{14} produced in the isocitritase reaction. Glyoxylic acid reductase is of fairly widespread occurrence in plants (Zelitch 1955, Zelitch and Ochoa 1953, and Stafford *et al*. 1954).

Production of glycollate-C^{14} with a very high specific activity is readily explainable on the basis of the glyoxylate cycle operation (see Scheme ii). Carbon from acetyl CoA enters the 3 and 4 positions of malate in the malate synthetase reaction. Following conversion to oxaloacetate and condensation with another molecule of acetyl CoA, the 4 and 3 positions of malate become the 1 and 2 positions of citrate and isocitrate. In the isocitritase reaction, these carbon atoms become the corresponding positions of the glyoxylate molecule. Clearly, if this glyoxylate or glycollate has a higher specific activity than malate, separation of the organic acids into storage and metabolic pools must occur *in vivo*.

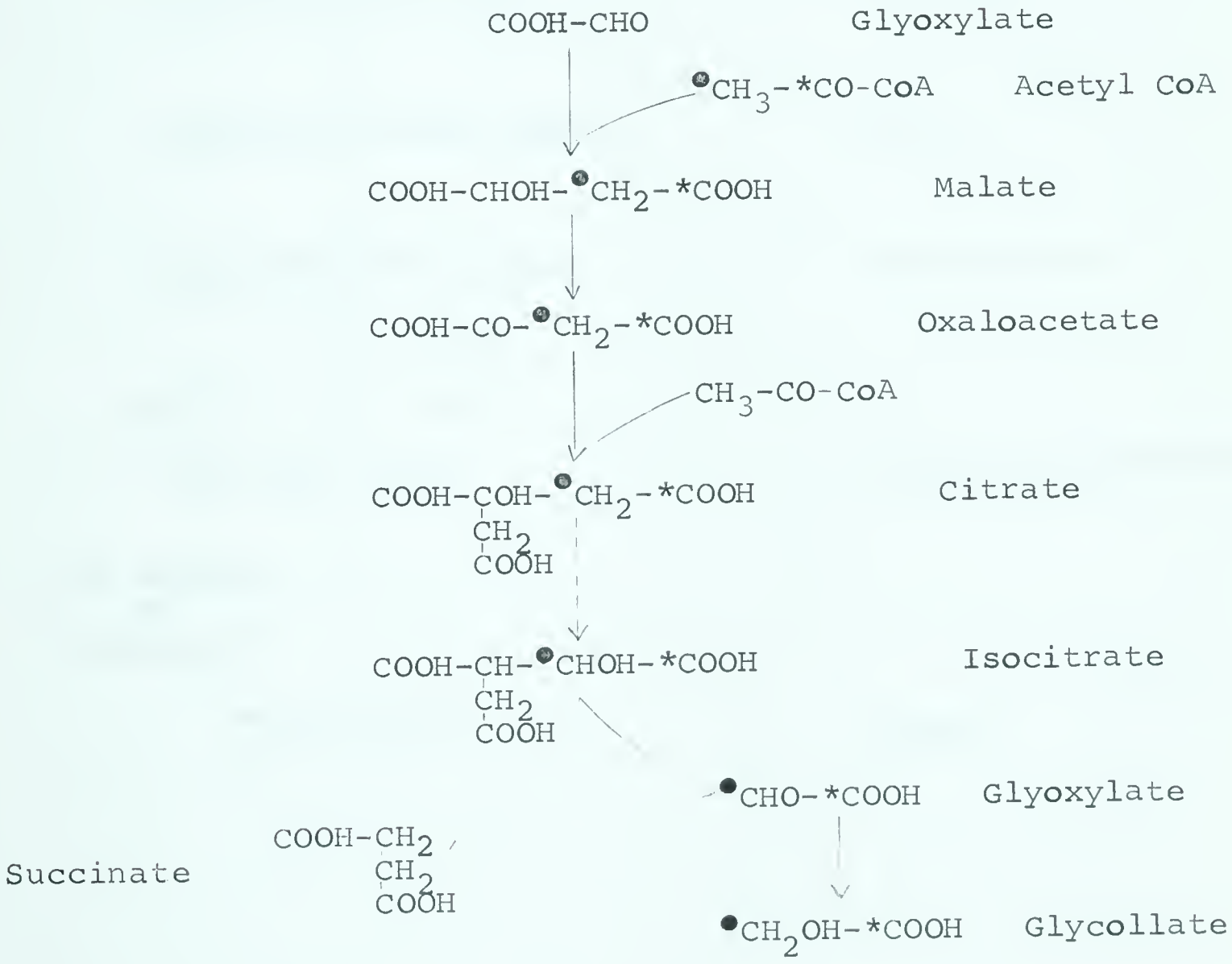
A further indication of the importance of the glyoxylate cycle in ethanol metabolism is evident from a comparison of the fates of the individual carbon atoms of the ethanol molecule. The origins of the carbon atoms incorporated into carbon dioxide and the sugars during operation of the glyoxylate cycle are shown in Scheme (iii). It is evident that utilization of acetyl-2-C^{14} CoA in the glyoxylate cycle

SCHEME (ii)

Conversion of Malate to Glycollate Via the Partial Reactions of the Glyoxylate Cycle

- Carbon atoms derived from the 2 position of acetyl CoA entering the cycle via malate synthetase.

* Carbon atoms derived from the 1 position of acetyl CoA via malate synthetase.

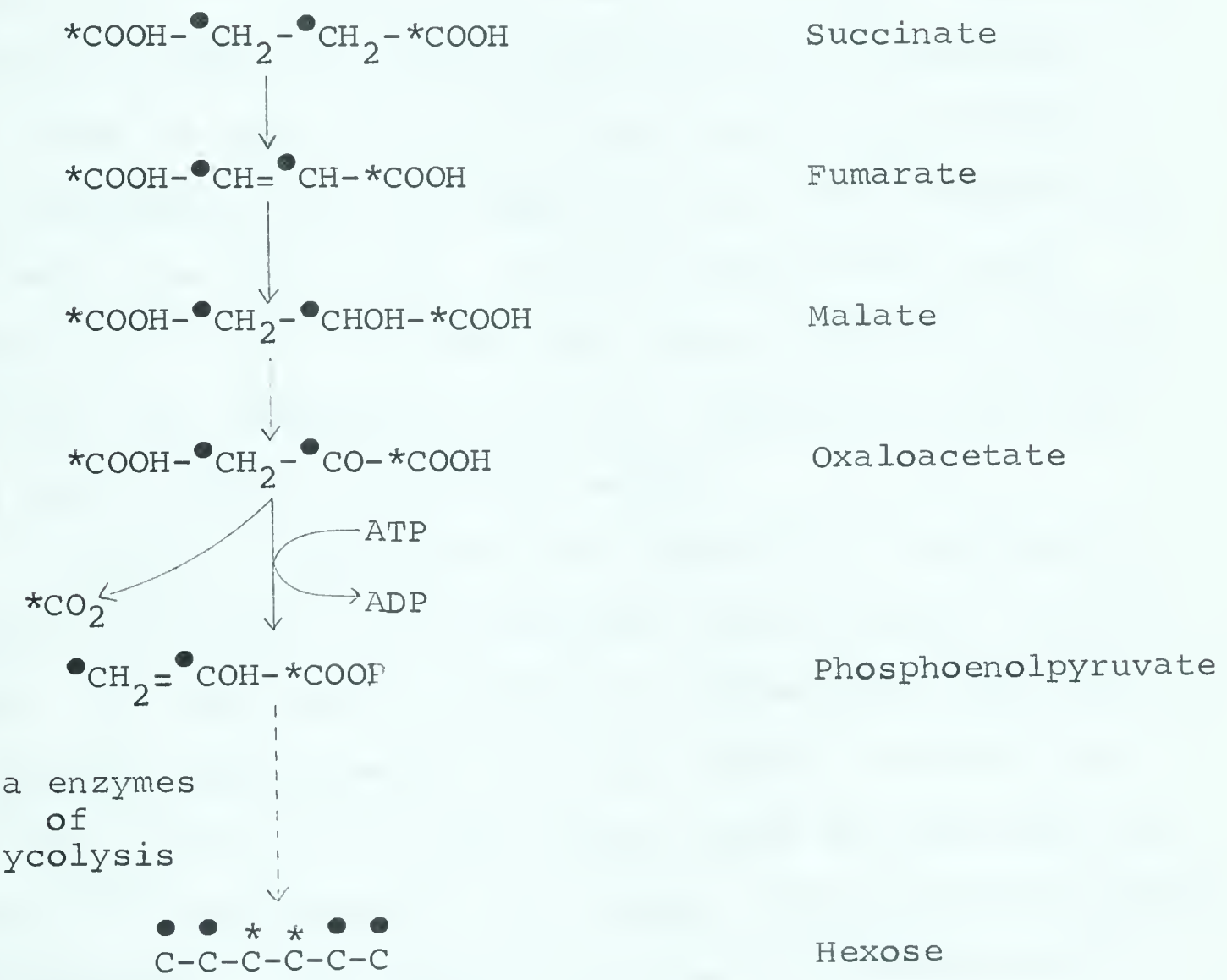


SCHEME (iii)

Conversion of Succinate to Phosphoenolpyruvate and Sugars

* Carbon atoms derived from carbon 1 of acetyl CoA.

• Carbon atoms derived from carbon 2 of acetyl CoA.



results in considerable incorporation of C^{14} into sugars. In contrast, utilization of acetyl-1-C^{14} CoA will result in production of labelled carbon dioxide. If ethanol is metabolized via acetyl CoA and the glyoxylate cycle, similar patterns of C^{14} incorporation should be evident. Figure 11 and Table X show that these predictions were fulfilled. Four times as much C^{14} from the two position of ethanol was incorporated into the sugars and only small amounts of C^{14} were given off as CO_2. From the experiments using ethanol-1-C^{14}, it is evident that large amounts of the one position were incorporated into the respired CO_2 and only very small amounts entered the sugars.

Succinic dehydrogenase was shown to be involved in ethanol metabolism in an experiment using malonate (Table XIII). The addition of malonate, a competitive inhibitor of succinic dehydrogenase activity, caused a build up of C^{14} from ethanol-C^{14} in the organic acids and decreased the total C^{14} incorporated by the tissues. The role of succinic dehydrogenase in the glyoxylate cycle is to convert the succinate to fumarate as the first step in the conversion of succinate to sugar (see Scheme iii). The inhibitory effects of malonate on ethanol metabolism are therefore consistent with the operation of the glyoxylate cycle.

THE ROLE OF THE TRICARBOXYLIC ACID CYCLE IN ETHANOL METABOLISM:

Enzymes necessary for the operation of the TCA cycle are known to be present in castor bean endosperm tissue

(Beevers and Walker 1956). It is possible, therefore, that some acetyl CoA entering the glyoxylate cycle might also be metabolized via the partial reactions of the TCA cycle. Results obtained by Canvin and Beevers (1961) as well as those presented in the Results section (Tables V, VI, X, XIII, and XVII) have clearly shown that the ratios of C^{14} entering CO_2 to that entering sugar in experiments using ethanol-1-C^{14} and acetate-1-C^{14} are always greater than 1:1. These findings therefore indicate that the one positions of ethanol and acetate are involved in reactions in addition to those for sugar biosynthesis as shown in Scheme (iii). As the CO_2 : sugar ratios are never unity, decarboxylation of the one positions of ethanol and acetate must involve enzyme systems in addition to PEP carboxykinase. Cycling of ethanol and acetate carbon through the TCA cycle would produce carbon dioxide as shown in Figure 11.

Another indication that ethanol and acetate metabolism involves the TCA cycle is shown by the production of glutamic acid from these substrates. Labelling of this amino acid can readily occur from α-ketoglutaric acid, one of the TCA cycle acids which is actually bypassed during operation of the glyoxylate cycle.

The ethanol-C^{14} and acetate-C^{14} feeding experiment using corn root tips, however, indicates that the main pathway for utilization of these compounds is the TCA cycle. These findings are in agreement with earlier work (Harley and Beevers

1963, Cossins and Beevers 1963). However in the present studies with castor bean endosperm, it is clear that the glyoxylate cycle plays a major role in the utilization of ethanol and acetate. In addition to this major pathway it must be stressed that the production of glutamic acid and the liberation of large amounts of CO_2 strongly suggest that part of the substrates are being metabolized by partial reactions of the TCA cycle which are not part of the glyoxylate cycle.

CONVERSION OF ETHANOL TO ACETYL CoA:

Since the glyoxylate and TCA cycles are apparently involved in the metabolism of ethanol in the tissues examined, it is likely that ethanol is converted to acetyl CoA prior to metabolism by these pathways. The mechanism by which this occurs has been extensively examined in rat liver. Most experimental work with this organ indicates that conversion of ethanol to acetate and to acetyl CoA occurs as shown by sequence (a) outlined on page 63. Lundquist *et al*. (1963) found that ethanol is converted almost quantitatively to acetate in this tissue. Evidence from inhibitor studies (Kinard *et al*. 1956 and Lundquist *et al*. 1963), from competition studies (Smith 1961) and from labelling ratio studies (Russell and van Bruggen 1964) point to the oxidation of ethanol to acetaldehyde and then to acetate in rat liver tissue. In the present study, evidence from the inhibition by iodoacetate of alcohol dehydrogenase *in vitro* and ethanol-C^{14}

incorporation *in vivo* shows that the major part, if not all of the ethanol metabolized by castor bean endosperm tissue, is oxidized to acetaldehyde by alcohol dehydrogenase. The subsequent fate of the acetaldehyde was investigated by isotopic competition and dilution studies. The presence of a large pool of acetate was shown to inhibit the incorporation of ethanol-C^{14} into the products but additions of ethanol did not influence acetate-C^{14} metabolism (Tables XIV, XV and XVI). These results indicate that ethanol is metabolized via acetate *in vivo*. The conversion of acetate to acetyl CoA in several plant tissues can be inferred from studies with sulphanilamide (Jones and Wignall 1955). An enzyme system capable of activating acetate has been detected in a variety of tissues (Millerd and Bonner 1954, Patrick 1957). The results of the present studies are therefore in agreement with conclusions drawn by Cossins and Beevers (1963), that the major pathway for ethanol metabolism in the endosperm tissue involves conversion to acetyl CoA.

DIFFERENCES IN ETHANOL AND ACETATE METABOLISM:

If ethanol carbon is entirely converted to acetate during its utilization, the final products of ethanol and acetate metabolism should be similar. This possibility was investigated in comparative studies of ethanol-C^{14} and acetate-C^{14} metabolism. An examination of tables XIX, XX, XVII and XVIII shows that differences in the utilization of ethanol and acetate are evident in both the 5 and 7 day-old

castor bean endosperm tissues. Acetate was more readily utilized than ethanol by the tissues. Thus when equimolar amounts of ethanol and acetate were fed during identical experimental periods, acetate carbon was more extensively metabolized. This could account for the greater incorporation of acetate into the organic acids, amino acids and sugars in the 7 day-old bean (Table XVII). However, the more rapid utilization of acetate does not necessarily explain the greater incorporation of ethanol-C^{14} into CO_2 and lipids. These differences indicate that ethanol may not be wholly converted to acetate during its utilization. A comparison of ethanol-C^{14} and acetate-C^{14} metabolism in the 5 day-old castor bean endosperm tissue (Table XVIII), also indicates that ethanol-C^{14} and acetate-C^{14} are not incorporated into the same final products. Tissues at this stage of germination were found to show maximal incorporation of ethanol-C^{14} (Table V). Also the rate of incorporation of ethanol in these 5 day-old tissues more nearly equals the rate of acetate incorporation. The most striking difference in ethanol and acetate utilization in the 5 day-old tissues, is the greater incorporation of acetate into sucrose in contrast to the large incorporation of ethanol into the organic acids. A possible reason for these differences could be the more extensive metabolism of acetate by the tissue. However, before definite conclusions can be drawn, more detailed research on this aspect of acetate and ethanol metabolism is required.

It is clear that differences between the products formed from ethanol and acetate were not entirely restricted to the castor bean tissues. As was shown in Table XXI, certain differences in the metabolism of these compounds was apparent in corn root tips. Again, ethanol-C^{14} was more readily incorporated into the lipids. This feature of ethanol metabolism is of interest as other workers (Cossins and Beevers 1962, and Castelfranco et al. 1963) have also reported on the conversion of ethanol to lipids. Similarly in animal tissues, prolonged metabolism of ethanol results in fat deposition. From this study it seems clear that the chief pathway for ethanol metabolism is oxidation to acetyl CoA via acetaldehyde and acetate. An explanation for the observed differences in ethanol and acetate metabolism might lie in the metabolic fate of acetaldehyde. Clearly, studies on the metabolism of acetaldehyde by these tissues might yield valuable information regarding the lipid forming tendency of ethanol.

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